SMRT® Tools Reference Guide

Introduction

This document describes the command-line tools included with SMRT Link v8.0. These tools are for use by bioinformaticians working with secondary analysis results.

- The command-line tools are located in the `$SMRT_ROOT/smrtlink/smrtcmds/bin` subdirectory.

Installation

The command-line tools are installed as an integral component of the SMRT Link software. For installation details, see **SMRT Link Software Installation (v8.0)**.

- To install **only** the command-line tools, use the `--smrttools-only` option with the installation command, whether for a new installation or an upgrade. Examples:

```
smrtlink-*.run --rootdir smrtlink --smrttools-only
smrtlink-*.run --rootdir smrtlink --smrttools-only --upgrade
```

Pacific Biosciences Command-Line Tools

Following is information on the Pacific Biosciences-supplied command-line tools included in the installation. Third-party tools installed are described at the end of the document.

Tool	Description
`bam2fasta/` `bam2fastq`	Converts PacBio® BAM files into gzipped FASTA and FASTQ files. See "bam2fasta/bam2fastq" on page 2.
`bamsieve`	Generates a subset of a BAM or PacBio Data Set file based on either a whitelist of hole numbers, or a percentage of reads to be randomly selected. See "bamsieve" on page 3.
`blasr`	Aligns long reads against a reference sequence. See "blasr" on page 5.
`ccs`	Calculates consensus sequences from multiple "passes" around a circularized single DNA molecule (SMRTbell® template). See "ccs" on page 10.
`dataset`	Creates, opens, manipulates and writes Data Set XML files. See "dataset" on page 15.
`Demultiplex` `Barcodes`	Identifies barcode sequences in PacBio single-molecule sequencing data. See "Demultiplex Barcodes" on page 21.
`gcpp`	Variant-calling tool which provides several variant-calling algorithms for PacBio sequencing data. See "gcpp" on page 32.
`ipdSummary`	Detects DNA base-modifications from kinetic signatures. See "ipdSummary" on page 34.

Tool	Description
isoseq3	Characterizes full-length transcripts and generates full-length transcript isoforms, eliminating the need for computational reconstruction. See "isoseq3" on page 38.
juliet	A general-purpose minor variant caller that identifies and phases minor single nucleotide substitution variants in complex populations. See "juliet" on page 41.
laa	Finds phased consensus sequences from a pooled set of amplicons sequenced with Pacific Biosciences' SMRT technology. See "laa" on page 49.
motifMaker	Identifies motifs associated with DNA modifications in prokaryotic genomes. See "motifMaker" on page 55.
pbalign	Aligns PacBio reads to reference sequences; filters aligned reads according to user-specified filtering criteria; and converts the output to PacBio BAM, SAM, or PacBio DataSet format. See "pbalign" on page 57.
pbcromwell	Pacific Biosciences' wrapper for the `cromwell` scientific workflow engine used to power SMRT Link. For details on how to use `pbcromwell` to run workflows, see "pbcromwell" on page 60.
pbdagcon	Implements DAGCon (Directed Acyclic Graph Consensus); a sequence consensus algorithm based on using directed acyclic graphs to encode multiple sequence alignments. See "pbdagcon" on page 63.
pbindex	Creates an index file that enables random access to PacBio-specific data in BAM files. See "pbindex" on page 64.
pbmm2	Aligns PacBio reads to reference sequences. A SMRT wrapper for `minimap2`, and the successor to `blasr` and `pbalign`. See "pbmm2" on page 65.
pbservice	Performs a variety of useful tasks within SMRT Link. See "pbservice" on page 71.
pbsv	Structural variant caller for PacBio reads. See "pbsv" on page 75.
pbvalidate	Validates that files produced by PacBio software are compliant with Pacific Biosciences' own internal specifications. See "pbvalidate" on page 79.
sawriter	Generates a suffix array file from an input FASTA file. See "sawriter" on page 81.
summarizeModifications	Generates a GFF summary file from the output of base modification analysis combined with the coverage summary GFF generated by resequencing pipelines. See "summarize Modifications" on page 82.

bam2fasta/ bam2fastq

The `bam2fastx` tools convert PacBio BAM files into gzipped FASTA and FASTQ files, including demultiplexing of barcoded data.

Usage

Both tools have an identical interface and take BAM and/or Data Set files as input.

Examples

```
bam2fasta -o projectName m54008_160330_053509.subreads.bam

bam2fastq -o myEcoliRuns m54008_160330_053509.subreads.bam
m54008_160331_235636.subreads.bam
```

```
bam2fasta -o myHumanGenomem54012_160401_000001.subreadset.xml
```

Input Files

- One or more `*.bam` files
- `*.subreadset.xml` file (Data Set file)

Output Files

- `*.fasta.gz`
- `*.fastq.gz`

bamsieve

The `bamsieve` tool creates a subset of a BAM or PacBio Data Set file based on either a whitelist of hole numbers, or a percentage of reads to be randomly selected, while keeping all subreads within a read together. Although `bamsieve` is BAM-centric, it has some support for dataset XML and will propagate metadata, as well as scraps BAM files in the special case of SubreadSets. `bamsieve` is useful for generating minimal test Data Sets containing a handful of reads.

`bamsieve` operates in two modes: **whitelist/blacklist** mode where the ZMWs to keep or discard are explicitly specified, or **percentage/count** mode, where a fraction of the ZMWs is randomly selected.

ZMWs may be whitelisted or blacklisted in one of several ways:

- As a comma-separated list on the command line.
- As a flat text file, one ZMW per line.
- As another PacBio BAM or Data Set of any type.

Usage

```
bamsieve [-h] [--version] [--log-file LOG_FILE]
                   [--log-level {DEBUG,INFO,WARNING,ERROR,CRITICAL} | --debug | --quiet
                   | -v]
                   [--show-zmws] [--whitelist WHITELIST] [--blacklist BLACKLIST]
                   [--percentage PERCENTAGE] [-n COUNT] [-s SEED]
                   [--ignore-metadata][--barcodes]
                   input_bam [output_bam]
```

Required	Description
`input_bam`	The name of the input BAM file or Data Set from which reads will be read.
`output_bam`	The name of the output BAM file or Data Set where filtered reads will be written to. (Default = `None`)

Options	Description
`-h`, `--help`	Displays help information and exits.
`--version`	Displays program version number and exits.
`--log-file LOG_FILE`	Writes the log to file. (Default = `None,` writes to `stdout`.)

Options	Description
`--log-level`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARNING`, `ERROR`, `CRITICAL]`. (Default = `WARNING`)
`--debug`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet`	Alias for setting the log level to `CRITICAL` to suppress output. (Default = `False`)
`-v, --verbose`	Sets the verbosity level. (Default = `NONE`)
`--show-zmws`	Prints a list of ZMWs and exits. (Default = `False`)
`--whitelist WHITELIST`	Specifies the ZMWs to **include** in the output. This can be a comma-separated list of ZMWs, or a file containing a list of ZMWs (one hole number per line), or a BAM/ Data Set file. (Default = `NONE`)
`--blacklist BLACKLIST`	Specifies the ZMWs to **exclude** from the output. This can be a comma-separated list of ZMWs, or a file containing a list of ZMWs (one hole number per line), or a BAM/Data Set file that specifies ZMWs. (Default = `NONE`)
`--percentage PERCENTAGE`	Specifies a percentage of a SMRT Cell to recover (Range = `1-100`) rather than a specific list of reads. (Default = `NONE`)
`-n COUNT, --count COUNT`	Specifies a specific number of ZMWs picked at random to recover. (Default = `NONE`)
`-s SEED, --seed SEED`	Specifies a random seed for selecting a percentage of reads. (Default = `NONE`)
`--ignore-metadata`	Discard the input Data Set metadata. (Default = `False`)
`--barcodes`	Specifies that the whitelist or blacklist contains barcode indices instead of ZMW numbers. (Default = `False`)

Examples

Pulling out two ZMWs from a BAM file:

```
$ bamsieve --whitelist 111111,222222 full.subreads.bam sample.subreads.bam
```

Pulling out two ZMWs from a Data Set file:

```
$ bamsieve --whitelist 111111,222222 full.subreadset.xml sample.subreadset.xml
```

Using a text whitelist:

```
$ bamsieve --whitelist zmws.txt full.subreads.bam sample.subreads.bam
```

Using another BAM or Data Set as a whitelist:

```
$ bamsieve --whitelist mapped.alignmentset.xml full.subreads.bam mappable.subreads.bam
```

Generating a whitelist from a Data Set:

```
$ bamsieve --show-zmws mapped.alignmentset.xml > mapped_zmws.txt
```

Anonymizing a Data Set:

```
$ bamsieve --whitelist zmws.txt --ignore-metadata --anonymize full.subreadset.xml
anonymous_sample.subreadset.xml
```

Removing a read:

```
$ bamsieve --blacklist 111111 full.subreadset.xml filtered.subreadset.xml
```

Selecting 0.1% of reads:

```
$ bamsieve --percentage 0.1 full.subreads.bam random_sample.subreads.bam
```

Selecting a different 0.1% of reads:

```
$ bamsieve --percentage 0.1 --seed 98765 full.subreads.bam random_sample.subreads.bam
```

Selecting just two ZMWs/reads at random:

```
$ bamsieve --count 2 full.subreads.bam two_reads.subreads.bam
```

Selecting by barcode:

```
$ bamsieve --barcodes --whitelist 4,7 full.subreads.bam two_barcodes.subreads.bam
```

Generating a tiny BAM file that contains only mappable reads:

```
$ bamsieve --whitelist mapped.subreads.bam full.subreads.bam mappable.subreads.bam
$ bamsieve --count 4 mappable.subreads.bam tiny.subreads.bam
```

Splitting a Data Set into two halves:

```
$ bamsieve --percentage 50 full.subreadset.xml split.1of2.subreadset.xml
$ bamsieve --blacklist split.1of2.subreadset.xml full.subreadset.xml
split.2of2.subreadset.xml
```

Extracting Unmapped Reads:

```
$ bamsieve --blacklist mapped.alignmentset.xml movie.subreadset.xml
unmapped.subreadset.xml
```

blasr The `blasr` tool aligns long reads against a reference sequence, possibly a multi-contig reference.

`blasr` maps reads to genomes by finding the highest scoring local alignment or set of local alignments between the read and the genome. The initial set of candidate alignments is found by querying a rapidly-searched precomputed index of the reference genome, and then refining until only high-scoring alignments are kept. The base assignment in alignments is optimized and scored using all available quality information, such as insertion and deletion quality values.

Because alignment approximates an exhaustive search, alignment significance is computed by comparing optimal alignment score to the distribution of all other significant alignment scores.

Usage

```
blasr {subreads|ccs}.bam genome.fasta --bam --out aligned.bam [--options]
blasr {subreadset|consensusreadset}.xml genome.fasta --bam --out aligned.bam [--
options]
blasr reads.fasta genome.fasta [--options]
```

Input Files

- `{subreads|ccs}.bam` is in PacBio BAM format, which is the native Sequel®/Sequel II System output format of SMRT reads. PacBio BAM files carry rich quality information (such as insertion, deletion, and substitution quality values) needed for mapping, consensus calling and variant detection. For the PacBio BAM format specifications, see http://pacbiofileformats.readthedocs.io/en/5.1/BAM.html.
- `{subreadset|consensusreadset}.xml` is in PacBio Data Set format. For the PacBio Data Set format specifications, see http://pacbiofileformats.readthedocs.io/en/5.1/DataSet.html.
- `reads.fasta`: A multi-FASTA file of reads. While any FASTA file is valid input, `bam` or `dataset` files are preferable as they contain more rich quality value information.
- `genome.fasta:` A FASTA file to which reads should map, usually containing reference sequences.

Output Files

- `aligned.bam:` The pairwise alignments for each read, in PacBio BAM format.

Input Options

Options	Description
`--sa suffixArrayFile`	Uses the suffix array `sa` for detecting matches between the reads and the reference. (The suffix array is prepared by the `sawriter` program.)
`--ctab tab`	Specifies a table of tuple counts used to estimate match significance, created by `printTupleCountTable`. While it is quick to generate on the fly, if there are many invocations of `blasr`, it is useful to precompute the `ctab`.
`--regionTable table`	Specifies a read-region table in HDF format for masking portions of reads. This may be a single table if there is just one input file, or a `fofn` (file-of-file names). When a region table is specified, any region table inside the `reads.plx.h5` or `reads.bax.h5` files is ignored. **Note**: This option works **only** with PacBio RS II HDF5 files.
`--noSplitSubreads`	Does **not** split subreads at adapters. This is typically only useful when the genome in an unrolled version of a known template, and contains template-adapter-reverse-template sequences. (Default = `False`)

Options for Aligning Output

Options	Description
`--bestn n`	Provides the top `n` alignments for the hit policy to select from. (Default = `10`)
`--sam`	Writes output in SAM format.
`--bam`	Writes output in PacBio BAM format.
`--clipping`	Uses no/hard/soft clipping for SAM output. (Default = `none`)
`--out file`	Writes output to `file`. (Default = `terminal`)
`--unaligned file`	Output reads that are **not** aligned to `file`.

Options	Description
`--m t`	If **not** printing SAM, modifies the output of the alignment. • t=0: Print `blast`-like output with |'s connecting matched nucleotides. • 1: Print only a summary: Score and position. • 2: Print in `Compare.xml` format. • 3: Print in vulgar format (**Deprecated**). • 4: Print a longer tabular version of the alignment. • 5: Print in a machine-parsable format that is read by `compareSequences.py`.
`--noSortRefinedAlignments`	Once candidate alignments are generated and scored via sparse dynamic programming, they are rescored using local alignment that accounts for different error profiles. Resorting based on the local alignment may change the order in which the hits are returned. (Default = `False`)
`--allowAdjacentIndels`	Allows adjacent insertion or deletions. Otherwise, adjacent insertion and deletions are merged into one operation. Using quality values to guide pairwise alignments may dictate that the higher probability alignment contains adjacent insertions or deletions. Tools such as GATK do **not** permit this and so they are not reported by default.
`--header`	Prints a header as the first line of the output file describing the contents of each column.
`--titleTable tab`	Builds a table of reference sequence titles. The reference sequences are enumerated by row, `0,1,...` The reference index is printed in alignment results rather than the full reference name. This makes output concise, particularly when very verbose titles exist in reference names. (Default = `NULL`)
`--minPctSimilarity p`	Reports alignments only if they are greater than `p` percent identity. (Default = `0`)
`--holeNumbers LIST`	Aligns reads whose ZMW hole numbers are in `LIST` **only**. `LIST` is a comma-delimited string of ranges, such as `1,2,3,10-13`. This option **only** works when reads are in base or pulse h5 format.
`--hitPolicy policy`	Specifies how `blasr` treats multiple hits: • `all`: Reports **all** alignments. • `allbest`: Reports all equally top-scoring alignments. • `random`: Reports a single random alignment. • `randombest`: Reports a single random alignment from multiple equally top-scoring alignments. • `leftmost:` Reports an alignment which has the best alignment score and has the smallest mapping coordinates in any reference.

Options for Anchoring Alignment Regions

- These options will have the greatest effects on speed and sensitivity.

Options	Description
`--minMatch m`	Specifies the minimum seed length. A higher value will speed up alignment, but decrease sensitivity. (Default = `12`)

`--maxMatch m`
`--maxLCPLength m`
	Stops mapping a read to the genome when the LCP length reaches `m`. This is useful when the query is part of the reference, for example when constructing pairwise alignments for *de novo* assembly. (Both options work the same.)

`--maxAnchorsPerPosition m`	Do **not** add anchors from a position if it matches to more than `m` locations in the target.
`--advanceExactMatches E`	Speeds up alignments with match `-E` fewer anchors. Rather than finding anchors between the read and the genome at every position in the read, when an anchor is found at position `i` in a read of length `L`, the next position in a read to find an anchor is at `i+L-E`. Use this when aligning already assembled contigs. (Default = `0`)

Options	Description
`--nCandidates n`	Keeps up to `n` candidates for the best alignment. A large value will slow mapping as the slower dynamic programming steps are applied to more clusters of anchors - this can be a rate-limiting step when reads are very long. (Default = `10`)
`--concordant`	Maps all subreads of a ZMW (hole) to where the longest full pass subread of the ZMW aligned to. This requires using the region table and hq regions. This option **only** works when reads are in base or pulse h5 format. (Default = `False`)
`--placeGapConsistently`	Produces alignments with gaps placed consistently for better variant calling. See "Gaps When Aligning" on page 10 for details.

Options for Refining Hits

Options	Description
`--refineConcordantAlignments`	Refines concordant alignments. This slightly increases alignment accuracy at the cost of time. This option is omitted if `--concordant` is **not** set to `True`. (Default = `False`)
`--sdpTupleSize K`	Uses matches of length `K` to speed dynamic programming alignments. This option controls accuracy of assigning gaps in pairwise alignments once a mapping has been found, rather than mapping sensitivity itself. (Default = `11`)

`--scoreMatrix "score matrix string"`	Specifies an alternative score matrix for scoring FASTA reads. The matrix is in the format
	`  ACGTN`
	`A abcde`
	`C fghij`
	`G klmno`
	`T pqrst`
	`N uvwxy`
	The values `a...y` should be input as a quoted space separated string: "`a b c ... y`". Lower scores are better, so matches should be less than mismatches; such as a,g,m,s = `-5` (match), mismatch = `6`.

`--affineOpen value`	Sets the penalty for opening an affine alignment. (Default = `10`)
`--affineExtend a`	Changes affine (extension) gap penalty. Lower value allows more gaps. (Default = `0`)

Options for Overlap/Dynamic Programming Alignments and Pairwise Overlap for *de novo* Assembly

Options	Description
`--useQuality`	Uses substitution/insertion/deletion/merge quality values to score gap and mismatch penalties in pairwise alignments. As the insertion and deletion rates are much higher than substitution, this makes many alignments favor an insertion/deletion over a substitution. Naive consensus-calling methods will then often miss substitution polymorphisms. Use this option when calling consensus using the Quiver method. **Note**: When **not** using quality values to score alignments, there will be a lower consensus accuracy in homopolymer regions. (Default = `False`)
`--affineAlign`	Refines alignment using affine guided align. (Default = `False`)

Options for Filtering Reads

Options	Description
--minReadLength l	Ignores reads that have a full length less than l. Subreads may be shorter. (Default = 50)
--minSubreadLength l	Does **not** align subreads of length less than l. (Default = 0)
--minAlnLength	Reports alignments **only** if their lengths are greater than this value. (Default = 0)

Options for Parallel Alignment

Options	Description
--nproc N	Aligns using N processes. All large data structures such as the suffix array and tuple count table are shared. (Default = 1)
--start S	Index of the first read to begin aligning. This is useful when multiple instances are running on the same data; for example when on a multi-rack cluster. (Default = 0)
--stride S	Aligns one read every S reads. (Default = 1)

Options for Subsampling Reads

Options	Description
--subsample p	Proportion p of reads to randomly subsample and align; expressed as a decimal. (Default = 0)
--help	Displays help information and exits.
--version	Displays version information using the format MajorVersion.Subversion.SHA1 (Example: 5.3.abcd123) and exits.

Examples

To align reads from reads.bam to the ecoli_K12 genome, and output in PacBio BAM format:

```
blasr reads.bam ecoli_K12.fasta --bam --out ecoli_aligned.bam
```

To use multiple threads:

```
blasr reads.bam ecoli_K12.fasta --bam --out ecoli_aligned.bam --proc 16
```

To include a larger minimal match, for faster but less sensitive alignments:

```
blasr reads.bam ecoli_K12.fasta --bam --out ecoli_aligned.bam --proc 16 --minMatch 15
```

To produce alignments in a pairwise human-readable format:

```
blasr reads.bam ecoli_K12.fasta -m 0
```

To use a precomputed suffix array for faster startup:

```
sawriter hg19.fasta.sa hg19.fasta #First precompute the suffix array
blasr reads.bam hg19.fasta --sa hg19.fasta.sa
```

Gaps When Aligning

By default, `blasr` places gap **inconsistently** when aligning a sequence and its reverse-complement sequence. It is preferable to place gap consistently to call a consensus sequence from multiple alignments or call single nucleotide variants (SNPs), as the output alignments will make it easier for variant callers to call variants.

Example:

```
REF  : TTTTTTAAACCCC
READ1: TTTTTTACCCC
READ2: GGGGTAAAAAA
```

where READ1 and READ2 are reverse-complementary to each other.

In the following alignments, gaps are placed **inconsistently**:

```
REF          : TTTTTTAAACCCC
READ1        : TTTTTTA--CCCC
RevComp(READ2): TTTTTT--ACCCC
```

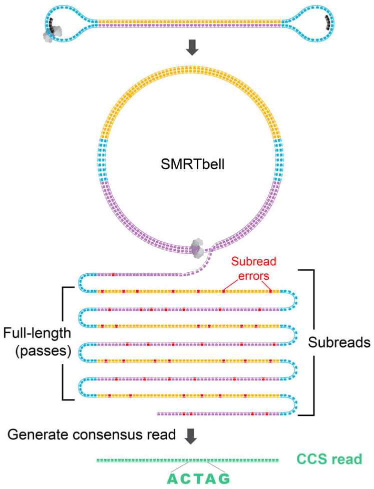
In the following alignments, gaps are placed **consistently**, with `--placeGapsConsistently` specified:

```
REF          : TTTTTTAAACCCC
READ1        : TTTTTTA--CCCC
RevComp(READ2): TTTTTTA--CCCC
```

To produce alignments with gaps placed **consistently** for better variant calling, use the `--placeGapConsistently` option:

```
blasr query.bam target.fasta --out outfile.bam --bam --placeGapConsistently
```

ccs Circular Consensus Sequencing (CCS) calculates consensus sequences from multiple “passes” around a circularized single DNA molecule (SMRTbell® template). CCS uses the Arrow framework to achieve optimal consensus results given the number of passes available.



Input Files

- One `.subreads.bam` file containing the subreads for each SMRTbell® template sequenced.

Output Files

- A BAM file with one entry for each consensus sequence derived from a ZMW. BAM is a general file format for storing sequence data, which is described fully by the SAM/BAM working group. The CCS output format is a version of this general format, where the consensus sequence is represented by the "Query Sequence". Several tags were added to provide additional meta information. An example BAM entry for a consensus as seen by `samtools` is shown below.

```
m141008_060349_42194_c100704972550000001823137703241586_s1_p0/63/ccs4*0255
**00CCCGGGGATCCTCTAGAATGC~~~~~~~~~~~~~~~~~~~~~RG:Z:83ba013f np:i:35 rq:f:0.999682
sn:B:f,11.3175,6.64119,11.6261,14.5199 zm:i:63
```

Following are some of the common fields contained in the output BAM file:

Field	Description
`Query Name`	Movie Name / ZMW # /ccs
`FLAG`	Required by the format but meaningless in this context. Always set to `4` to indicate the read is unmapped.
`Reference Name`	Required by the format but meaningless in this context. Always set to `*`.
`Mapping Start`	Required by the format but meaningless in this context. Always set to `0`.
`Mapping Quality`	Required by the format but meaningless in this context. Always set to `255`.
`CIGAR`	Required by the format but meaningless in this context. Always set to `*`.
`RNEXT`	Required by the format but meaningless in this context. Always set to `*`.
`PNEXT`	Required by the format but meaningless in this context. Always set to `0`.
`TLEN`	Required by the format but meaningless in this context. Always set to `0`.

Field	Description
`Consensus Sequence`	The consensus sequence generated.
`Quality Values`	The per-base parametric quality metric. For details see "Interpreting QUAL Values" on page 13.
`RG Tag`	The read group identifier.
`bc Tag`	A 2-entry array of upstream-provided barcode calls for this ZMW.
`bq Tag`	The quality of the barcode call. (**Optional**: Depends on barcoded inputs.)
`np Tag`	The number of full passes that went into the subread. (**Optional**: Depends on barcoded inputs.)
`rq Tag`	The predicted read quality.
`t2 Tag`	The time (in seconds) spent aligning subreads to the draft consensus, prior to polishing.
`t3 Tag`	The time (in seconds) spent polishing the draft consensus, not counting retries.
`zm Tag`	The ZMW hole number.

Usage

```
ccs [OPTIONS] INPUT OUTPUT
```

Example

```
ccs --minlength 100 myData.subreads.bam myResult.bam
```

Required	Description
`Input File Name`	The name of a single `subreads.bam` or a `subreadset.xml` file to be processed. (Example = `myData.subreads.bam`)
`Output File Name`	The name of the output BAM file; comes after all other options listed. Valid output files are the BAM and the Dataset .xml formats. (Example = `myResult.bam`)

Options	Description
`--version`	Prints the version number.
`--report-file`	Contains a result tally of the outcomes for all ZMWs that were processed. If **no** file name is given, the report is output to the file `ccs_report.txt` In addition to the count of successfully-produced consensus sequences, this file lists how many ZMWs failed various data quality filters (SNR too low, not enough full passes, and so on) and is useful for diagnosing unexpected drops in yield.
`--min-snr`	Removes data that is likely to contain deletions. SNR is a measure of the strength of signal for all 4 channels (A, C, G, T) used to detect base pair incorporation. This value sets the threshold for minimum required SNR for any of the four channels. Data with SNR < 2.5 is typically considered lower quality. (Default = `2.5)`
`--min-length`	Specifies the minimum length requirement for the minimum length of the draft consensus to be used for further polishing. If the targeted template is known to be a particular size range, this can filter out alternative DNA templates. (Default = `10`)
`--max-length`	Specifies the maximum length requirement for the maximum length of the draft consensus to be used for further polishing. For robust results while avoiding unnecessary computation on unusual data, set to ~20% above the largest expected insert size. (Default = `50000`)

Options	Description
`--min-passes`	Specifies the minimum number of passes for a ZMW to be emitted. This is the number of full passes. Full passes **must** have an adapter hit before and after the insert sequence and so do **not** include any partial passes at the start and end of the sequencing reaction. It is computed as the number of passes mode across all windows. (Default = `3`)
`--min-rq`	Specifies the minimum predicted accuracy of a read. `ccs` generates an accuracy prediction for each read, defined as the expected percentage of matches in an alignment of the consensus sequence to the true read. A value of `0.99` indicates that only reads expected to be 99% accurate are emitted. (Default = `0.99`)
`--num-threads`	Specifies how many threads to use while processing. By default, `ccs` will use as many threads as there are available cores to minimize processing time, but fewer threads can be specified here.
`--log-file`	The name of a log file to use. If none is given, the logging information is printed to `STDERR`. (Example: `mylog.txt`)
`--log-level`	Specifies verbosity of log data to produce. By setting `--logLevel=DEBUG`, you can obtain detailed information on what ZMWs were dropped during processing, as well as any errors which may have appeared. (Default = `INFO`)
`--skip-polish`	After constructing the draft consensus, do **not** proceed with the polishing steps. This is significantly faster, but generates less accurate data with no RQ or QUAL values associated with each base.
`--by-strand`	Separately generates a consensus sequence from the forward and reverse strands. Useful for identifying heteroduplexes formed during sample preparation.
`--chunk`	Operates on a single chunk. Format `i/N`, where `i` in [1,N]. Examples: `3/24` or `9/9`.
`--max-chunks`	Determines the maximum number of chunks, given an input file.
`--modelPath`	Specifies the path to a model file or directory containing model files.
`--modelSpec`	Specifies the name of the chemistry or model to use, overriding the default selection.

Interpreting QUAL Values

The QUAL value of a read is a measure of the posterior likelihood of an error at a particular position. **Increasing** QUAL values are associated with a **decreasing** probability of error. For indels and homopolymers, there is ambiguity as to which QUAL value is associated with the error probability. Shown below are different types of alignment errors, with a `*` indicating which sequence BP should be associated with the alignment error.

Mismatch

```
           *
    ccs: ACGTATA
    ref: ACATATA
```

Deletion

```
           *
    ccs: AC-TATA
    ref: ACATATA
```

Insertion

```
       *
ccs: ACGTATA
ref: AC-TATA
```

Homopolymer Insertion or Deletion

Indels should always be left-aligned, and the error probability is only given for the first base in a homopolymer.

```
       *                    *
ccs: ACGGGGTATA     ccs: AC-GGGTATA
ref: AC-GGGTATA     ref: ACGGGGTATA
```

CCS Yield Report

The CCS Report specifies the number of ZMWs that successfully produced consensus sequences, as well as a count of how many ZMWs did **not** produce a consensus sequence for various reasons. The entries in this report, as well as parameters used to increase or decrease the number of ZMWs that pass various filters, are shown in the table below.

The first part is a summary of inputs and outputs:

ZMW Results	Parameters Affecting Results	Description
ZMWs input (A)	None	The number of input ZMWs.
ZMWs generating CCS (B)	All custom processing settings	The number of CCS reads successfully produced on the first attempt, using the fast windowed approach.
ZMWs filtered (C)	All custom processing settings	The number of ZMWs reads that failed producing a CCS read.

The second part explains in details the exclusive ZMW count for (C), those ZMWs that were filtered:

ZMW Results	Parameters Affecting Results	Description

No usable subreads	`--minReadScore,`
	`--minLength,`
	`--maxLength`
		The ZMW had no usable subreads. Either there were no subreads, or all subreads had lengths outside the range <50% or >200% of the median subread length.

Below SNR threshold	`--min-snr`	The ZMW had at least one channel's SNR below the minimum threshold.
Lacking full passes	`--min-passes`	There were not enough subreads that had an adapter at the start and end of the subread (a "full pass").
Heteroduplexes	None	The SMRTbell contains a heteroduplex. In this case, it is not clear what the consensus should be and so the ZMW is dropped.
Min coverage violation	None	The ZMW is damaged on one strand and cannot be polished reliably.

ZMW Results	Parameters Affecting Results	Description
Draft generation error	None	Subreads do not match the generated draft sequence, even after multiple tries.
Draft above --max-length	`--max-length`	The draft sequence was above the maximum length threshold.
Draft below --min-length	`--min-length`	The draft sequence was below the minimum length threshold.
Lacking usable subreads	None	Too many subreads were dropped while polishing
CCS did not converge	None	The consensus sequence did not converge after the maximum number of allowed rounds of polishing.
CCS below minimum predicted accuracy	`--min-rq`	Each CCS read has a predicted level of accuracy associated with it. Reads that are below the minimum specified threshold are removed.
Unknown error during processing	None	These should not occur.

dataset

The `dataset` tool creates, opens, manipulates and writes Data Set XML files. The commands allow you to perform operations on the various types of data held by a Data Set XML: Merge, split, write, and so on.

Usage

```
dataset [-h] [--version] [--log-file LOG_FILE]
        [--log-level {DEBUG,INFO,WARNING,ERROR,CRITICAL} | --debug | --quiet | -v]
        [--strict] [--skipCounts]

{create,filter,merge,split,validate,summarize,consolidate,loadstats,newuuid,loadmetada
ta,copyto,absolutize,relativize}
```

Options	Description
`-h, --help`	Displays help information and exits.
`<Command> -h`	Displays help for a specific command.
`-v, --version`	Displays program version number and exits.
`--log-file LOG_FILE`	Writes the log to file. (Default = `None`, writes to `stdout`.)
`--log-level`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARNING`, `ERROR`, `CRITICAL]`. (Default = `INFO`)
`--debug`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet`	Alias for setting the log level to `CRITICAL` to suppress output. (Default = `False`)
`-v`	Sets the verbosity level. (Default = `NONE`)
`--strict`	Turns on strict tests and display **all** errors. (Default = `False`)
`--skipCounts`	Skips updating `NumRecords` and `TotalLength` counts. (Default = `False`)

`create` Command: Create an XML file from a `fofn` (file-of-file names) or BAM file. Possible types: `SubreadSet, AlignmentSet, ReferenceSet,` HdfSubreadSet, BarcodeSet, ConsensusAlignmentSet, ConsensusReadSet, ContigSet.

```
dataset create [-h] [--type DSTYPE] [--name DSNAME] [--generateIndices]
               [--metadata METADATA] [--novalidate] [--relative]
               outfile infile [infile ...]
```

Example

The following example shows how to use the `dataset create` command to create a barcode file:

```
$ dataset create --generateIndices --name my_barcodes --type BarcodeSet
my_barcodes.barcodeset.xml my_barcodes.fasta
```

Required	Description
`outfile`	The name of the XML file to create.
`infile`	The `fofn` (file-of-file-names) or BAM file(s) to convert into an XML file.

Options	Description
`--type DSTYPE`	Specifies the type of XML file to create. (Default = `NONE`)
`--name DSNAME`	The name of the new Data Set XML file.
`--generateIndices`	Generates index files (`.pbi` and `.bai` for BAM, `.fai` for FASTA). Requires `samtools`/`pysam` and `pbindex`. (Default = `FALSE`)
`--metadata METADATA`	A `metadata.xml` file (or Data Set XML) to supply metadata. (Default = `NONE`)
`--novalidate`	Specifies **not** to validate the resulting XML. Leaves the paths as they are.
`--relative`	Makes the included paths relative instead of absolute. This is **not** compatible with `--novalidate`.

`filter` Command: Filter an XML file using filters and threshold values.

- Suggested filters: `accuracy`, `bc`, `bcf`, `bcq`, `bcr`, `bq`, `cx`, `length`, `movie`, `n_subreads`, `pos`, `qend`, `qname`, `qstart`, `readstart`, `rname`, `rq`, `tend`, `tstart`, `zm`.
- More resource-intensive filter: [`qs`]

Note: Multiple filters with different names are ANDed together. Multiple filters with the **same** name are ORed together, duplicating existing requirements.

```
dataset filter [-h] infile outfile filters [filters ...]
```

Required	Description
`infile`	The name of the XML file to filter.
`outfile`	The name of the output filtered XML file.

Required	Description
filters	The values to filter on. (Example: rq>0.85)

merge Command: Combine XML files.

```
dataset merge [-h] outfile infiles [infiles ...]
```

Required	Description
infiles	The names of the XML files to merge.
outfile	The name of the output XML file.

split Command: Split a Data Set XML file.

```
dataset split [-h] [--contigs] [--barcodes] [--zmws] [--byRefLength]
                   [--noCounts] [--chunks CHUNKS] [--maxChunks MAXCHUNKS]
                   [--targetSize TARGETSIZE] [--breakContigs]
                   [--subdatasets] [--outdir
                   infile [outfiles...]
```

Required	Description
infile	The name of the XML file to split.

Options	Description
outfiles	The names of the resulting XML files.
--contigs	Splits the XML file based on contigs. (Default = FALSE)
--barcodes	Splits the XML file based on barcodes. (Default = FALSE)
--zmws	Splits the XML file based on ZMWs. (Default = FALSE)
--byRefLength	Splits contigs by contig length. (Default = TRUE)
--noCounts	Updates the Data Set counts after the split. (Default = FALSE)
--chunks x	Splits contigs into x total windows. (Default = 0)
--maxChunks x	Splits the contig list into at most x groups. (Default = 0)
--targetSize x	Specifies the minimum number of records per chunk. (Default = 5000)
--breakContigs	Breaks contigs to get closer to maxCounts. (Default = False)
--subdatasets	Splits the XML file based on subdatasets. (Default = False)
--outdir OUTDIR	Specifies an output directory for the resulting XML files. (Default = <in-place>, **not** the current working directory.)

validate Command: Validate XML and ResourceId files. (This is an internal testing functionality that may be useful.)

Note: This command requires that pyxb (**not** distributed with SMRT Link) be installed. If **not** installed, validate simply checks that the files pointed to in ResourceIds exist.

```
dataset validate [-h] [--skipFiles] infile
```

Required	Description
`infile`	The name of the XML file to validate.

Options	Description
`--skipFiles`	Skips validating external resources. (Default = `False`)

`summarize` Command: Summarize a Data Set XML file.

```
dataset summarize [-h] infile
```

Required	Description
`infile`	The name of the XML file to summarize.

`consolidate` Command: Consolidate XML files.

```
dataset consolidate [-h] [--numFiles NUMFILES] [--noTmp]
                    infile datafile xmlfile
```

Required	Description
`infile`	The name of the XML file to consolidate.
`datafile`	The name of the resulting data file.
`xmlfile`	The name of the resulting XML file.

Options	Description
`--numFiles x`	Specifies the number of data files to produce. (Default = `1`)
`--noTmp`	Do **not** copy to a temporary location to ensure local disk use. (Default = `False`)

`loadstats` Command: Load an `sts.xml` file containing pipeline statistics into a Data Set XML file.

```
dataset loadstats [-h] [--outfile OUTFILE] infile statsfile
```

Required	Description
`infile`	The name of the Data Set XML file to modify.
`statsfile`	The name of the `.sts.xml` file to load.

Options	Description
`--outfile OUTFILE`	The name of the XML file to output. (Default = `None`)

`newuuid` Command: Refresh a Data Set's Unique ID.

```
dataset newuuid [-h] [--random] infile
```

Required	Description
`infile`	The name of the XML file to refresh.

Options	Description
`--random`	Generates a random UUID, instead of a hash. (Default = `False`)

`loadmetadata` Command: Load a `.metadata.xml` file into a Data Set XML file.

```
dataset loadmetadata [-h] [--outfile OUTFILE] infile metadata
```

Required	Description
`infile`	The name of the Data Set XML file to modify.
`metadata`	The `.metadata.xml` file to load, or Data Set to borrow from.

Options	Description
`--outfile OUTFILE`	Specifies the XML file to output. (Default = `None`)

`copyto` Command: Copy a Data Set and resources to a new location.

```
dataset copyto [-h] [--relative] infile outdir
```

Required	Description
`infile`	The name of the XML file to copy.
`outdir`	The directory to copy to.

Options	Description
`--relative`	Makes the included paths relative instead of absolute. (Default = `False`)

`absolutize` Command: Make the paths in an XML file absolute.

```
dataset absolutize [-h] [--outdir OUTDIR] infile
```

Required	Description
`infile`	The name of the XML file whose paths should be absolute.

Options	Description
`--outdir OUTDIR`	Specifies an optional output directory. (Default = `None`)

`relativize` Command: Make the paths in an XML file relative.

```
dataset relativize [-h] infile
```

Required	Description
`infile`	The name of the XML file whose paths should be relative.

Example - Filter Reads

To filter one or more BAM file's worth of subreads, aligned or otherwise, and then place them into a single BAM file:

```
# usage: dataset filter <in_fn.xml> <out_fn.xml> <filters>
dataset filter in_fn.subreadset.xml filtered_fn.subreadset.xml 'rq>0.85'

# usage: dataset consolidate <in_fn.xml> <out_data_fn.bam> <out_fn.xml>
dataset consolidate filtered_fn.subreadset.xml consolidate.subreads.bam
out_fn.subreadset.xml
```

The filtered Data Set and the consolidated Data Set should be read-for-read equivalent when used with SMRT® Analysis software.

Example - Resequencing Pipeline

- Align two movie's worth of subreads in two SubreadSets to a reference.
- Merge the subreads together.
- Split the subreads into Data Set chunks by contig.
- Process using `gcpp` on a chunkwise basis (in parallel).

1. Align each movie to the reference, producing a Data Set with one BAM file for each execution:

```
pbalign movie1.subreadset.xml referenceset.xml movie1.alignmentset.xml
pbalign movie2.subreadset.xml referenceset.xml movie2.alignmentset.xml
```

2. Merge the files into a FOFN-like Data Set; BAMs are **not** touched:

```
# dataset merge <out_fn> <in_fn> [<in_fn> <in_fn> ...]
dataset merge merged.alignmentset.xml movie1.alignmentset.xml movie2.alignmentset.xml
```

3. Split the Data Set into chunks by contig name; BAMs are **not** touched:
   - Note that supplying output files splits the Data Set into that many output files (up to the number of contigs), with multiple contigs per file.
   - **Not** supplying output files splits the Data Set into **one** output file per contig, named automatically.
   - Specifying a number of chunks instead will produce that many files, with contig or even subcontig (reference window) splitting.

```
dataset split --contigs --chunks 8 merged.alignmentset.xml
```

4. Process the chunks:

```
gcpp --reference referenceset.xml --output
chunk1consensus.fasta,chunk1consensus.fastq,chunk1consensus.vcf,chunk1consensus.gff
chunk1contigs.alignmentset.xml
```

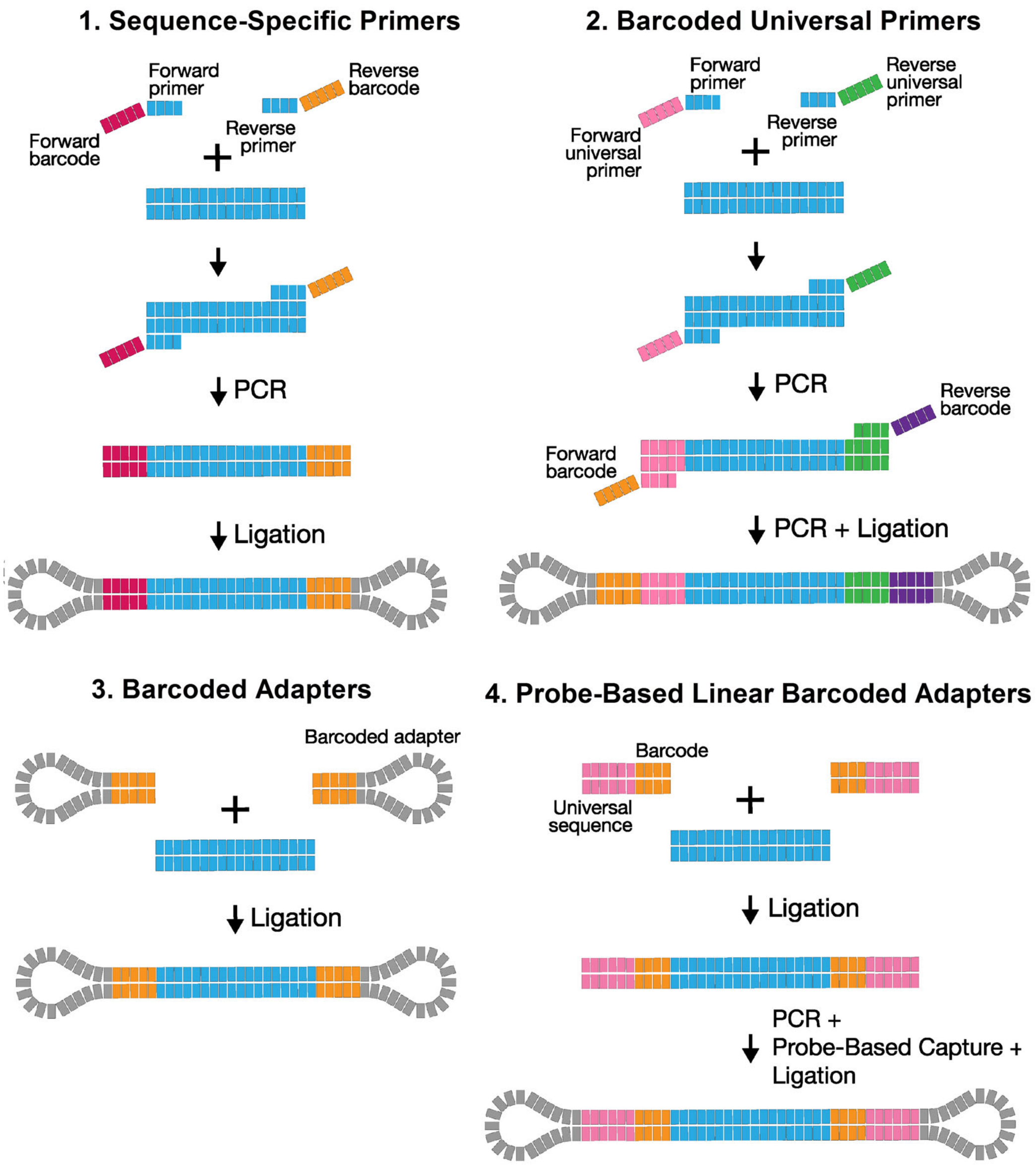
The chunking works by duplicating the original merged Data Set (no BAM duplication) and adding filters to each duplicate such that only reads belonging to the appropriate contigs are emitted. The contigs are distributed among the output files in such a way that the total number of records per chunk is about even.

Demultiplex Barcodes

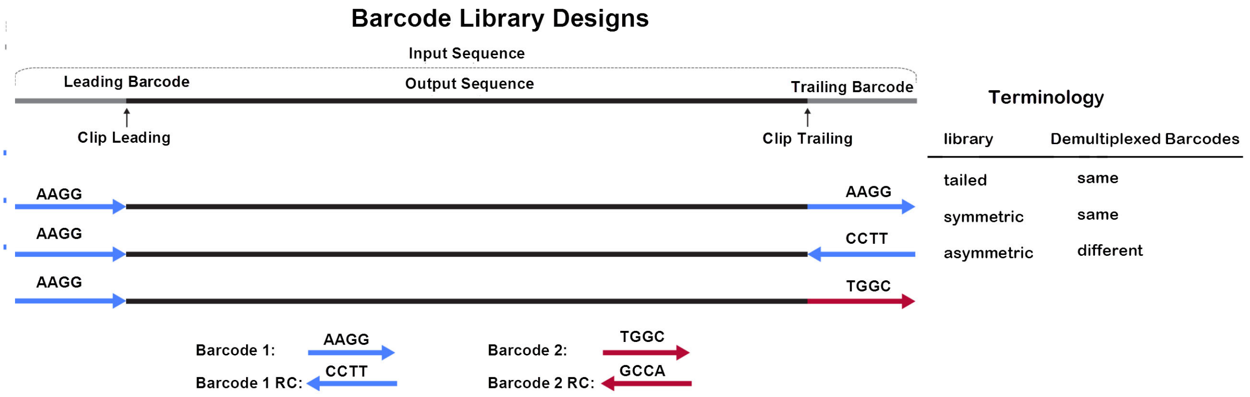
The **Demultiplex Barcodes** application identifies barcode sequences in PacBio single-molecule sequencing data. It **replaced** `pbbarcode` and `bam2bam` for demultiplexing, starting with SMRT® Analysis v5.1.0.

Demultiplex Barcodes can demultiplex samples that have a unique per-sample barcode pair and were pooled and sequenced on the same SMRT Cell. There are four different methods for barcoding samples with PacBio technology:

1. Sequence-specific primers
2. Barcoded universal primers
3. Barcoded adapters
4. Linear Barcoded Adapters for Probe-based Captures



In addition, there are three different barcode library designs. As **Demultiplex Barcodes** supports raw subread and CCS read demultiplexing, the following terminology is based on the per (sub-) read view.



In the overview above, the input sequence is flanked by adapters on both sides. The bases adjacent to an adapter are **barcode regions**. A read can have up to two barcode regions, leading and trailing. Either or both adapters can be missing and consequently the leading and/or trailing region is not being identified.

For **symmetric** and **tailed** library designs, the **same** barcode is attached to both sides of the insert sequence of interest. The only difference is the orientation of the trailing barcode. For barcode identification, one read with a single barcode region is sufficient.

For the **asymmetric** design, **different** barcodes are attached to the sides of the insert sequence of interest. To identify the different barcodes, a read with leading and trailing barcode regions is required.

Output barcode pairs are generated from the identified barcodes. The barcode names are combined using “`--`“, for example `bc1002--bc1054`. The sort order is defined by the barcode indices, starting with the lowest.

Workflow

By default, **Demultiplex Barcodes** processes input reads grouped by ZMW, **except** if the `--per-read` option is used. All barcode regions along the read are processed individually. The final per-ZMW result is a summary over all barcode regions. Each ZMW is assigned to a pair of selected barcodes from the provided set of candidate barcodes. Subreads from the same ZMW will have the same barcode and barcode quality. For a particular target barcode region, every barcode sequence gets aligned as given and as reverse-complement, and higher scoring orientation is chosen. This results in a list of scores over all candidate barcodes.

- If only **same** barcode pairs are of interest (symmetric/tailed), use the `--same` option to filter out **different** barcode pairs.
- If only **different** barcode pairs are of interest (asymmetric), use the `--different` option to require at least two barcodes to be read, and remove pairs with the **same** barcode.

Half Adapters

For an adapter call with only one barcode region, the high-quality region finder cuts right through the adapter. The preceding or succeeding subread was too short and was removed, or the sequencing reaction started/stopped there. This is called a **half adapter**. Thus, there are also 1.5, 2.5, N+0.5 adapter calls.

ZMWs with half or only one adapter can be used to identify the same barcode pairs; positive-predictive value might be reduced compared to high adapter calls. For asymmetric designs with different barcodes in a pair, at least a single full-pass read is required; this can be two adapters, two half adapters, or a combination.

Usage:

- Any existing output files are **overwritten** after execution.
- Always use `--peek-guess` to remove spurious barcode hits.

Analysis of subread data:

```
lima movie.subreads.bam barcodes.fasta prefix.bam
lima movie.subreadset.xml barcodes.barcodeset.xml prefix.subreadset.xml
```

Analysis of CCS data:

```
lima --css movie.ccs.bam barcodes.fasta prefix.bam
lima --ccs movie.consensusreadset.xml barcodes.barcodeset.xml
prefix.consensusreadset.xml
```

If you do not need to import the demultiplexed data into SMRT Link, use the `--no-pbi` option to minimize memory consumption and run time.

Symmetric or Tailed options:

```
Raw: --same
CCS: --same --ccs
```

Asymmetric options:

```
Raw: --different
CCS: --different --ccs
```

Example Execution:

```
lima m54317_180718_075644.subreadset.xml \
Sequel_RSII_384_barcodes_v1.barcodeset.xml \
m54317_180718_075644.demux.subreadset.xml \
--different --peek-guess
```

Options	Description
`--same`	Retains only reads with the **same** barcodes on both ends of the insert sequence, such as symmetric and tailed designs.
`--different`	Retains only reads with **different** barcodes on both ends of the insert sequence, asymmetric designs. Enforces `--min-passes ≥ 1`.

Options	Description
`--min-length n`	Omits reads with lengths below `n` base pairs after demultiplexing. ZMWs with no reads passing are omitted. (Default = `50`)
`--max-input-length n`	Omits reads with lengths above `n` base pairs for scoring in the demultiplexing step. (Default = `0`, deactivated)
`--min-score n`	Omits ZMWs with average barcode scores below `n`. A **barcode score** measures the alignment between a barcode attached to a read and an ideal barcode sequence, and is an indicator how well the chosen barcode pair matches. It is normalized to a range between `0` (no hit) and `100` (a perfect match). (Default = `0,` Pacific Biosciences recommends setting it to `26`.)
`--min-end-score n`	Specifies the minimum end barcode score threshold applied to the individual leading and trailing ends. (Default = `0`)

`--min-passes n`	Omits ZMWs with less than `n` full passes, a read with a leading and trailing adapter. (Default = `0`, no full-pass needed) Example:
	`0 pass  : insert - adapter - insert`
	`1 pass  : insert - adapter - INSERT - adapter - insert`
	`2 passes: insert - adapter - INSERT - adapter - INSERT - adapter - insert`

`--score-full-pass`	Uses only reads flanked by adapters on both sides (full-pass reads) for barcode identification.
`--min-ref-span`	Specifies the minimum reference span relative to the barcode length. (Default = `0.5`)
`--per-read`	Scores and tags per subread, instead of per ZMW.
`--ccs`	Sets defaults to `-A 1 -B 4 -D 3 -I 3 -X 1`.
`--peek n`	Looks at the first `n` ZMWs of the input and return the mean. This lets you test multiple test `barcode.fasta` files and see which set of barcodes was used.
`--guess n`	This performs demultiplexing twice. In the first iteration, **all** barcodes are tested per ZMW. Afterwards, the barcode occurrences are counted and their mean is tested against the threshold `n`; only those barcode pairs that pass this threshold are used in the second iteration to produce the final demultiplexed output. A `prefix.lima.guess` file shows the decision process; `--same` is being respected.
`--guess-min-count`	Specifies the minimum ZMW count to whitelist a barcode. This filter is ANDed with the minimum barcode score specified by `--guess`. (Default = `0`)
`--peek-guess`	Equivalent to the **Infer Barcodes Used** parameter option in SMRT Link. Sets the following options: `--peek 50000 --guess 45 --guess-min-count 10.` Demultiplex Barcodes will run twice on the input data. For the first 50,000 ZMWs, it will guess the barcodes and store the mask of identified barcodes. In the second run, the barcode mask is used to demultiplex all ZMWs. If combined with `--ccs` then the barcode score threshold is increased by `--guess 75`.
`--single-side`	Identifies barcodes in molecules that only have barcodes adjacent to one adapter.
`--window-size-mult` `--window-size-bp`	The candidate region size multiplier: `barcode_length * multiplier.` (Default = `3`) Optionally, you can specify the region size in base pairs using `--window-size-bp`. If set, `--window-size-mult` is ignored.

Options	Description
`--num-threads n`	Spawns `n` threads; `0` means use all available cores. This option also controls the number of threads used for BAM and PBI compression. (Default = `0`)
`--chunk-size n`	Specifies that each thread consumes `n` ZMWs per chunk for processing. (Default = `10`).
`--no-bam`	Does **not** produce BAM output. Useful if only reports are of interest, as run time is shorter.
`--no-pbi`	Does **not** produce a `.bam.pbi` index file. The on-the-fly `.bam.pbi` file generation buffers the output data. If you do not need a `.bam.pbi` index file for SMRT Link import, use this option to decrease memory usage to a minimum and shorten the run time.
`--no-reports`	Does **not** produce any reports. Useful if only demultiplexed BAM files are needed.
`--dump-clips`	Outputs all clipped barcode regions generated to the `<prefix>.lima.clips` file.
`--dump-removed`	Outputs all records that did **not** pass the specified thresholds, or are without barcodes, to the `<prefix>.lima.removed.bam` file.
`--split-bam` `--split-bam-named`	Specifies that each barcode has its own BAM file called `prefix.idxBest-idxCombined.bam`, such as `prefix.0-0.bam`. Optionally ,`--split-bam-named` names the files by their barcode names instead of their barcode indices.
`--isoseq`	Removes primers as part of the Iso-Seq pipeline. See "Demultiplexing Iso-Seq Data" on page 31 for details.
`--bad-adapter-ratio n`	Specifies the maximum ratio of bad adapters. (Default = `0`).

Input Files:

Input data in PacBio-enhanced BAM format is either:

- Sequence data - Unaligned subreads, directly from a Sequel/Sequel II System, or
- Unaligned CCS reads, generated by CCS 2.

Note: To demultiplex PacBio RS II data, use SMRT Link or `bax2bam` to convert `.h5` files to BAM format.

Barcodes are provided as a FASTA file or BarcodeSet file:

- One entry per barcode sequence.
- **No** duplicate sequences.
- All bases must be in **upper-case**.
- Orientation-agnostic (forward or reverse-complement, but **not** reversed.)

Example:

```
>bc1000
CTCTACTTACTTACTG
```

```
>bc1001
GTCGTATCATCATGTA
>bc1002
AATATACCTATCATTA
```

Note: Name barcodes using an alphabetic character prefix to avoid later barcode name/index confusion.

Output Files:

Demultiplex Barcodes generates multiple output files by default, all starting with the same prefix as the output file, using the suffixes `.bam`, `.subreadset.xml`, and `.consensusreadset.xml`. The report prefix is `lima`. Example:

```
lima m54007_170702_064558.subreads.bam barcode.fasta /my/path/
m54007_170702_064558_demux.subreadset.xml
```

For all output files, the prefix is `/my/path/m54007_170702_064558_demux`.

- `<prefix>.bam`: Contains clipped records, annotated with barcode tags, that passed filters and respect the `--same` option.
- `<prefix>.lima.report`: A tab-separated file describing each ZMW, unfiltered. This is useful information for investigating the demultiplexing process and the underlying data. A single row contains **all** reads from a single ZMW. For `--per-read`, each row contains one subread, and ZMWs might span multiple rows.
- `<prefix>.lima.summary`: Lists how many ZMWs were filtered, how many ZMWs are the same or different, and how many reads were filtered.

```
(1)
ZMWs input                (A) : 213120
ZMWs above all thresholds (B) : 176356 (83%)
ZMWs below any threshold  (C) : 36764 (17%)

(2)
ZMW Marginals for (C) :
Below min length             : 26 (0%)
Below min score              : 0 (0%)
Below min end score          : 5138 (13%)
Below min passes             : 0 (0%)
Below min score lead         : 11656 (32%)
Below min ref span           : 3124 (8%)
Without adapter              : 25094 (68%)
With bad adapter             : 10349 (28%) <- Only with --bad-adapter-ratio
Undesired hybrids            : xxx (xx%) <- Only with --peek-guess
Undesired same barcode pairs : xxx (xx%) <- Only with --different
Undesired diff barcode pairs : xxx (xx%) <- Only with --same
Undesired 5p--5p pairs       : xxx (xx%) <- Only with --isoseq
Undesired 3p--3p pairs       : xxx (xx%) <- Only with --isoseq
Undesired single side        : xxx (xx%) <- Only with --isoseq
Undesired no hit             : xxx (xx%) <- Only with --isoseq
```

(3)

```
ZMWs for (B):
With same barcode            : 162244 (92%)
With different barcodes      : 14112 (8%)
Coefficient of correlation   : 32.79%
```

(4)

```
ZMWs for (A):
Allow diff barcode pair      : 157264 (74%)
Allow same barcode pair      : 188026 (88%)
Bad adapter yield loss       : 10112 (5%) <- Only with --bad-adapter-ratio
Bad adapter impurity         : 10348 (5%) <- Only without --bad-adapter-ratio
```

(5)

```
Reads for (B):
Above length                 : 1278461 (100%)
Below length                 : 2787 (0%)
```

Explanation of each block:

1. Number of ZMWs that went into `lima`, how many ZMWs were passed to the output file, and how many did not qualify.
2. For those ZMWs that did not qualify: The marginal counts of each filter. (Filter are described in the **Options** table.)
   When running with `--peek-guess` or similar manual option combination and different barcode pairs are found during peek, the full SMRT Cell may contain low-abundant different barcode pairs that were identified during peek individually, but **not** as a pair. Those unwanted barcode pairs are called **hybrids**.
3. For those ZMWs that passed: How many were flagged as having the same or different barcode pair, as well as the coefficient of variation for the barcode ZMW yield distribution in percent.
4. For all input ZMWs: How many allow calling the same or different barcode pair. This is a simplified version of how many ZMW have at least one full pass to allow a different barcode pair call and how many ZMWs have at least half an adapter, allowing the same barcode pair call.
5. For those ZMWs that qualified: The number of reads that are above and below the specified `--min-length` threshold.

- `<prefix>.lima.counts`: A `.tsv` file listing the counts of each observed barcode pair. Only passing ZMWs are counted. Example:

```
$ column -t prefix.lima.counts
```

IdxFirst	IdxCombined	IdxFirstNamed	IdxCombinedNamed	Counts	MeanScore
0	0	bc1001	bc1001	1145	68
1	1	bc1002	bc1002	974	69
2	2	bc1003	bc1003	1087	68

- `<prefix>.lima.clips`: Contains clipped barcode regions generated using the `--dump-clips` option. Example:

```
$ head -n 6 prefix.lima.clips
>m54007_170702_064558/4850602/6488_6512 bq:34 bc:11
CATGTCCCCTCAGTTAAGTTACAA
>m54007_170702_064558/4850602/6582_6605 bq:37 bc:11
TTTTGACTAACTGATACCAATAG
>m54007_170702_064558/4916040/4801_4816 bq:93 bc:10
```

- `<prefix>.lima.removed.bam`: Contains records that did **not** pass the specified thresholds, or are without barcodes, using the option `--dump-removed`.

  `lima` does **not** generate a `.pbi`, nor Data Set for this file. This option **cannot** be used with any splitting option.
- `<prefix>.lima.guess`: A `.tsv` file that describes the barcode subsetting process activated using the `--peek` and `--guess` options.

IdxFirst	IdxCombined	IdxFirstNamed	IdxCombinedNamed	NumZMWs	MeanScore	Picked
0	0	bc1001t	bc1001t	1008	50	1
1	1	bc1002t	bc1002t	1005	60	1
2	2	bc1003t	bc1003t	5	24	0
3	3	bc1004t	bc1004t	555	61	1

- One DataSet,`.subreadset.xml`, or `.consensusreadset.xml` file is generated per output BAM file.
- `.pbi`: One PBI file is generated per output BAM file.

What is a universal spacer sequence and how does it affect demultiplexing?

For library designs that include an identical sequence between adapter and barcode, such as probe-based linear barcoded adapters samples, Demultiplex Barcodes offers a special mode that is activated if it finds a shared prefix sequence among all provided barcode sequences.

Example:

```
>custombc1
ACATGACTGTGACTATCTCACACATATCAGAGTGCG
>custombc2
ACATGACTGTGACTATCTCAACACACAGACTGTGAG
```

In this case, Demultiplex Barcodes detects the shared prefix `ACATGACTGTGACTATCTCA` and removes it internally from all barcodes. Subsequently, it increases the window size by the length `L` of the prefix sequence.

- If `--window-size-bp N` is used, the actual window size is `L + N`.

- If `--window-size-mult M` is used, the actual window size is `(L + |bc|) * M`.

Because the alignment is semi-global, a leading reference gap can be added without any penalty to the barcode score.

What are bad adapters?

In the `subreads.bam` file, each subread has a context flag `cx`. The flag specifies, among other things, whether a subread has flanking adapters, before and/or after. Adapter-finding was improved and can also find molecularly-missing adapters, or those obscured by a local decrease in accuracy. This may lead to missing or obscured bases in the flanking barcode. Such adapters are labelled "bad", as they don't align with the adapter reference sequence(s). Regions flanking those bad adapters are problematic, because they can fully or partially miss the barcode bases, leading to wrong classification of the molecule. `lima` can handle those adapters by **ignoring** regions flanking bad adapters. For this, `lima` computes the ratio of number of bad adapters divided by number of all adapters.

By default, `--bad-adapter-ratio` is set to `0` and does **not** perform any filtering. In this mode, bad adapters are handled just like good adapters.

But the `*.lima.summary` file contains one row with the number of ZMWs that have at least 25% bad adapters, but otherwise pass all other filters. This metric can be used as a diagnostic to assess library preparation.

If `--bad-adapter-ratio` is set to non-zero positive `(0,1)`, bad adapter flanking barcode regions are treated as missing. If a ZMW has a higher ratio of bad adapters than provided, the ZMW is filtered and consequently removed from the output. The `*.lima.summary` file contains two additional rows.

```
With bad adapter        : 10349 (28%)
Bad adapter yield loss  : 10112 (5%)
```

The first row counts the number of ZMWs that have bad adapter ratios that are too high; the percentage is with respect to the number of all ZMW not passing. The second row counts the number of ZMWs that are removed solely due to bad adapter ratios that are too high; the percentage is with respect the number of all input ZMWs and consequently is the effective yield loss caused by bad adapters.

If a ZMW has ~50% bad adapters, one side of the molecule is molecularly-missing an adapter. For 100% bad adapter, **both** sides are missing adapters. A lower than ~40% percentage indicates decreased local accuracy during sequencing leading to adapter sequences not being found. If a high percentage of ZMWs is molecularly-missing adapters, you should improve library preparation.

Demultiplexing Iso-Seq Data

Demultiplex Barcodes is used to identify and remove Iso-Seq cDNA primers. If the Iso-Seq sample is barcoded, the barcodes should be included as part of the primer. **Note**: To demultiplex Iso-Seq samples in the SMRT Link (GUI), **always** choose the Iso-Seq Analysis or Iso-Seq Analysis with Mapping applications, **not** the Demultiplex Barcodes application. Only by using the command line can users use `lima` with the `--isoseq` option for demultiplexing Iso-Seq data.

The input Iso-Seq data format for demultiplexing is `.ccs.bam`. Users must first generate a CCS BAM file for an Iso-Seq Data Set before running `lima`. The recommended parameters for running CCS for Iso-Seq are `min-pass=1`, `min accuracy=0.8`, and turning Polish to OFF.

1. Primer IDs must be specified using the suffix `_5p` to indicate 5' cDNA primers and the suffix `_3p` to indicate 3' cDNA primers. The 3' cDNA primer should **not** include the Ts and is written in reverse complement.
2. Below are two example primer sets. The first is **unbarcoded**, the second has barcodes (shown in lower case) adjacent to the 3' primer.

   Example 1: The IsoSeq v2 primer set.

   ```
   >NEB_5p
   GCAATGAAGTCGCAGGGTTGGG
   >Clontech_5p
   AAGCAGTGGTATCAACGCAGAGTACATGGGG
   >NEB_Clontech_3p
   GTACTCTGCGTTGATACCACTGCTT
   ```

   Example 2: 4 tissues were multiplexed using barcodes on the 3' end only.

   ```
   >5p
   AAGCAGTGGTATCAACGCAGAGTACATGGGG
   >tissue1_3p
   atgacgcatcgtctgaGTACTCTGCGTTGATACCACTGCTT
   >tissue2_3p
   gcagagtcatgtatagGTACTCTGCGTTGATACCACTGCTT
   >tissue3_3p
   gagtgctactctagtaGTACTCTGCGTTGATACCACTGCTT
   >tissue4_3p
   catgtactgatacacaGTACTCTGCGTTGATACCACTGCTT
   ```

3. Use the `--isoseq` mode. Note that this **cannot** be combined with the `--guess` option.
4. The output will be only different pairs with a 5p and 3p combination:

   ```
   demux.5p--tissue1_3p.bam
   demux.5p--tissue2_3p.bam
   ```

The `--isoseq` parameter set is very conservative for removing any spurious and ambiguous calls, and guarantees that only proper asymmetric (barcoded) primer are used in downstream analyses. Good libraries reach >75% CCS reads passing the Demultiplex Barcodes filters.

gcpp

gcpp is a variant-calling tool provided by the GCpp package which provides several variant-calling algorithms for PacBio sequencing data.

Usage

```
gcpp    -j8 --algorithm=arrow \
        -r lambdaNEB.fa        \
        -o variants.gff        \
        aligned_subreads.bam
```

This example requests variant-calling, using 8 worker processes and the Arrow algorithm, taking input from the file aligned_subreads.bam, using the FASTA file lambdaNEB.fa as the reference, and writing output to variants.gff.

A particularly useful option is --referenceWindow/-w; which allows the variant-calling to be performed exclusively on a **window** of the reference genome.

Input Files

- A sorted file of reference-aligned reads in Pacific Biosciences' standard BAM format.
- A FASTA file that follows the Pacific Biosciences FASTA file convention.

Note: The --algorithm=arrow option requires that certain metrics be in place in the input BAM file. It requires per-read SNR metrics, and the per-base PulseWidth metric for Sequel data.

The selected algorithm will stop with an error message if any features that it requires are unavailable.

Output Files

Output files are specified as comma-separated arguments to the -o flag. The file name extension provided to the -o flag is meaningful, as it determines the output file format. For example:

```
gcpp aligned_subreads.bam -r lambda.fa  -o myVariants.gff,myConsensus.fasta
```

will read input from aligned_subreads.bam, using the reference lambda.fa, and send variant call output to the file myVariants.gff, and consensus output to myConsensus.fasta.

The file formats currently supported (using extensions) are:

- .gff: PacBio GFFv3 variants format; convertible to BED.
- .vcf: VCF 4.2 variants format (that is compatible with v4.3.)
- .fasta: FASTA file recording the consensus sequence calculated for each reference contig.

- `.fastq`: FASTQ file recording the consensus sequence calculated for each reference contig, as well as per-base confidence scores.

Options	Description
`-j`	Specifies the number of worker processes to use.
`--algorithm=`	Specifies the variant-calling algorithm to use; values are `plurality` and `arrow`.
`-r`	Specifies the FASTA reference file to use.
`-o`	Specifies the output file format; values are `.gff`, `.vcf`, `.fasta`, and `.fastq`.
`--maskRadius`	When using the `arrow` algorithm, setting this option to a value `N` greater than 0 causes `gcpp` to pass over the data a second time after masking out regions of reads that have >70% errors in 2*N+1 bases. This setting has little to no effect at low coverage, but for high-coverage datasets (>50X), setting this parameter to `3` may improve final consensus accuracy. In rare circumstances, such as misassembly or mapping to the wrong reference, enabling this parameter **may** cause worse performance.

`--minConfidence MINCONFIDENCE`
`-q MINCONFIDENCE`
	Specifies the minimum confidence for a variant call to be output to variants.{gff,vcf} (Default = `40`)

`--minCoverage MINCOVERAGE`
`-x MINCOVERAGE`
	Specifies the minimum site coverage for variant calls and consensus to be calculated for a site. (Default = `5`)

Available Algorithms

At this time there are three algorithms available for variant calling: `plurality`, `poa` and `arrow`.

- `plurality` is a simple and very fast procedure that merely tallies the most frequent read base or bases found in alignment with each reference base, and reports deviations from the reference as potential variants. This is a very insensitive and flawed approach for PacBio sequence data, and is prone to insertion and deletion errors.
- `poa` uses the partial order alignment algorithm to determine the consensus sequence. It is a heuristic algorithm that approximates a multiple sequence alignment by progressively aligning sequences to an existing set of alignments.
- `arrow` uses the per-read SNR metric and the per-pulse `pulsewidth` metric as part of its likelihood model.

Confidence Values

The `arrow` and `plurality` algorithms make a confidence metric available for every position of the consensus sequence. The confidence should be interpreted as a phred-transformed posterior probability that the consensus call is incorrect; such as:

$$QV = -10\log_{10}(p_{err})$$

`gcpp` clips reported QV values at 93; larger values **cannot** be encoded in a standard FASTQ file.

Chemistry Specificity

The `--algorithm=arrow` parameter is trained per-chemistry. `arrow` identifies the sequencing chemistry used for each run by looking at metadata contained in the input BAM data file. This behavior can be overridden by a command-line option.

When multiple chemistries are represented in the reads in the input file, the Arrow will model reads appropriately using the parameter set for its chemistry, thus yielding optimal results.

ipdSummary

The `ipdSummary` tool detects DNA base-modifications from kinetic signatures. It is part of the `kineticsTool` package.

`kineticsTool` loads IPDs observed at each position in the genome, compares those IPDs to value expected for unmodified DNA, and outputs the result of this statistical test. The expected IPD value for unmodified DNA can come from either an in-silico control or an amplified control. The in-silico control is trained by Pacific Biosciences and shipped with the package. It predicts the IPD using the local sequence context around the current position. An amplified control Data Set is generated by sequencing unmodified DNA with the same sequence as the test sample. An amplified control sample is usually generated by whole-genome amplification of the original sample.

Modification Detection

The basic mode of `kineticsTool` does an independent comparison of IPDs at each position on the genome, for each strand, and outputs various statistics to CSV and GFF files (after applying a significance filter).

Modifications Identification

`kineticsTool` also has a Modification Identification mode that can decode multi-site IPD “fingerprints” into a reduced set of calls of specific modifications. This feature has the following benefits:

- Different modifications occurring on the same base can be distinguished; for example, 6mA and 4mC.
- The signal from one modification is combined into one statistic, improving sensitivity, removing extra peaks, and correctly centering the call.

Algorithm: Synthetic Control

Studies of the relationship between IPD and sequence context reveal that most of the variation in mean IPD across a genome can be predicted from a 12-base sequence context surrounding the active site of the DNA polymerase. The bounds of the relevant context window correspond to the window of DNA in contact with the polymerase, as seen in DNA/ polymerase crystal structures. To simplify the process of finding DNA modifications with PacBio data, the tool includes a pre-trained lookup table mapping 12-mer DNA sequences to mean IPDs observed in C2 chemistry.

Algorithm: Filtering and Trimming

`kineticsTool` uses the Mapping QV generated by `blasr` and stored in the `cmp.h5` or BAM file (or AlignmentSet) to **ignore** reads that are not confidently mapped. The default minimum Mapping QV required is 10, implying that `blasr` has 90% confidence that the read is correctly mapped. Because of the range of read lengths inherent in PacBio data, this can be changed using the `--mapQvThreshold` option.

There are a few features of PacBio data that require special attention to achieve good modification detection performance. `kineticsTool` inspects the alignment between the observed bases and the reference sequence for an IPD measurement to be included in the analysis. The PacBio read sequence **must** match the reference sequence for `k` around the cognate base. In the current module, `k=1`. The IPD distribution at some locus can be thought of as a mixture between the “normal” incorporation process IPD, which is sensitive to the local sequence context and DNA modifications, and a contaminating “pause” process IPD, which has a much longer duration (mean > 10 times longer than normal), but happen rarely (~1% of IPDs).
Note: Our current understanding is that pauses do **not** carry useful information about the methylation state of the DNA; however a more careful analysis may be warranted. Also note that modifications that drastically increase the roughly 1% of observed IPDs are generated by pause events. Capping observed IPDs at the global 99th percentile is motivated by theory from robust hypothesis testing. Some sequence contexts may have naturally longer IPDs; to avoid capping too much data at those contexts, the cap threshold is adjusted per context as follows:

```
capThreshold = max(global99, 5*modelPrediction,
percentile(ipdObservations, 75))
```

Algorithm: Statistical Testing

We test the hypothesis that IPDs observed at a particular locus in the sample have longer means than IPDs observed at the same locus in unmodified DNA. If we have generated a Whole Genome Amplified Data Set, which removes DNA modifications, we use a case-control, two-sample t-test. This tool also provides a pre-calibrated “synthetic control” model which predicts the unmodified IPD, given a 12-base sequence context. In the synthetic control case we use a one-sample t-test, with an adjustment to account for error in the synthetic control model.

Usage

To run using a BAM input, and output GFF and HDF5 files:

```
ipdSummary aligned.bam --reference ref.fasta  m6A,m4C --gff basemods.gff \
--csv_h5 kinetics.h5
```

To run using `cmp.h5` input, perform methyl fraction calculation, and output GFF and CSV files:

```
ipdSummary aligned.cmp.h5 --reference ref.fasta  m6A,m4C --methylFraction \
--gff basemods.gff --csv kinetics.csv
```

Output Options	Description
`--gff FILENAME`	GFF format.
`--csv FILENAME`	Comma-separated value format.
`--csv_h5 FILENAME`	Compact binary-equivalent of .csv, in HDF5 format.
`--bigwig FILENAME`	BigWig file format; mostly only useful for SMRT View.

Input Files

- A standard PacBio alignment file - either AlignmentSet XML, BAM, or `cmp.h5` - containing alignments and IPD information.
- Reference sequence used to perform alignments. This can be either a FASTA file or a ReferenceSet XML.

Output Files

The tool provides results in a variety of formats suitable for in-depth statistical analysis, quick reference, and consumption by visualization tools such as SMRT View. Results are generally indexed by reference position and reference strand. In all cases the strand value refers to the strand carrying the modification in the DNA sample. Remember that the kinetic effect of the modification is observed in read sequences aligning to the opposite strand. So reads aligning to the positive strand carry information about modification on the negative strand and vice versa, but the strand containing the putative modification is always reported.

- `modifications.gff`: Compliant with the GFF Version 3 specification (http://www.sequenceontology.org/gff3.shtml). Each template position/strand pair whose probability value exceeds the probability value threshold appears as a row. The template position is 1-based, per the GFF specifications. The strand column refers to the strand carrying the detected modification, which is the opposite strand from those used to detect the modification. The GFF confidence column is a Phred-transformed probability value of detection.

  The auxiliary data column of the GFF file contains other statistics which may be useful for downstream analysis or filtering. These include the coverage level of the reads used to make the call, and +/- 20 bp sequence context surrounding the site.

- `modifications.csv`: Contains one row for each (reference position, strand) pair that appeared in the Data Set with coverage at least `x`. `x` defaults to `3`, but is configurable with the `--minCoverage` option. The reference position index is 1-based for compatibility with the GFF file in the R environment. Note that this output type scales poorly and is **not** recommended for large genomes; the HDF5 output should perform much better in these cases.

Output Columns: In-Silico Control Mode

Column	Description
`refId`	Reference sequence ID of this observation.
`tpl`	1-based template position.
`strand`	Native sample strand where kinetics were generated. `0` is the strand of the original FASTA, `1` is opposite strand from FASTA.
`base`	The cognate base at this position in the reference.
`score`	Phred-transformed probability value that a kinetic deviation exists at this position.
`tMean`	Capped mean of normalized IPDs observed at this position.
`tErr`	Capped standard error of normalized IPDs observed at this position (`standard deviation/sqrt(coverage)`).
`modelPrediction`	Normalized mean IPD predicted by the synthetic control model for this sequence context.
`ipdRatio`	`tMean/modelPrediction`.
`coverage`	Count of valid IPDs at this position.
`frac`	Estimate of the fraction of molecules that carry the modification.
`fracLow`	2.5% confidence bound of the `frac` estimate.
`fracUpp`	97.5% confidence bound of the `frac` estimate.

Output Columns: Case Control Mode

Column	Description
`refId`	Reference sequence ID of this observation.
`tpl`	1-based template position.
`strand`	Native sample strand where kinetics were generated. `0` is the strand of the original FASTA, `1` is opposite strand from FASTA.
`base`	The cognate base at this position in the reference.
`score`	Phred-transformed probability value that a kinetic deviation exists at this position.
`caseMean`	Mean of normalized case IPDs observed at this position.
`controlMean`	Mean of normalized control IPDs observed at this position.
`caseStd`	Standard deviation of case IPDs observed at this position.
`controlStd`	Standard deviation of control IPDs observed at this position.
`ipdRatio`	`tMean/modelPrediction`.
`testStatistic`	T-test statistic.

Column	Description
`coverage`	Mean of case and control coverage.
`controlCoverage`	Count of valid control IPDs at this position.
`caseCoverage`	Count of valid case IPDs at this position.

isoseq3

The `isoseq3` tool characterizes full-length transcripts. The analysis is performed *de novo*, without a reference genome. The tool enables analysis and functional characterization of transcript isoforms for sequencing data generated on PacBio instruments.

Usage

```
isoseq3 <tool>
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits

Typical workflow

1. Generate consensus sequences from raw subread data:

```
$ ccs movie.subreads.bam movie.ccs.bam --noPolish --minPasses 1
```

2. Remove primers and demultiplex:

```
$ cat primers.fasta
>primer_5p
AAGCAGTGGTATCAACGCAGAGTACATGGGG
>primer_3p
AAGCAGTGGTATCAACGCAGAGTAC
$ lima movie.ccs.bam primers.fasta demux.ccs.bam --isoseq --no-pbi
```

3. Remove noise from FL reads:

```
$ isoseq3 refine movie.fl.P5--P3.bam primers.fasta movie.flnc.bam
```

4. Cluster consensus sequences to generate unpolished transcripts:

```
$ isoseq3 cluster movie.flnc.bam unpolished.bam --verbose
```

5. Polish transcripts using subreads:

```
$ isoseq3 polish unpolished.bam movie.subreads.bam polished.bam
```

6. (**Optional**) Map transcripts to genome and collapse transcripts based on genomic mapping:

```
$ pbmm2 align polished.bam reference.fasta aligned.sorted.bam --preset ISOSEQ --sort
$ isoseq3 collapse aligned.sorted.bam out.gff or
$ isoseq3 collapse aligned.sorted.bam movie.ccs.bam out.gff
```

`cluster` Tool: Cluster CCS reads and generate unpolished transcripts.

Usage

```
isoseq3 cluster [options] input output
```

Example

```
isoseq3 cluster movie.consensusreadset.xml unpolished.bam
```

Custom BAM Tags

isoseq3 adds the following custom PacBio tags to the output BAM file:

- ib: Barcode summary - triplets delimited by semicolons; each triplet contains two barcode indices and the ZMW counts; delimited by comma. Example: 0,1,20;0,3,5
- im: ZMW names associated with this isoform.
- is: Number of ZMWs associated with this isoform.
- iz: Maximum number of subreads used for polishing.
- rq: Predicted accuracy for polished isoform.

Inputs/Outputs	Description
input	ccs.bam file or movie.consensusreadset.xml file.
output	unpolished.bam file or unpolished.transcriptset.xml file.

Options	Description
--require-polya	Requires full-length reads to have a poly(A) tail and removes it.
--s1	Specifies the number of seeds for minimer-only clustering. (Default = 1000)
--s2	Specifies the number of seeds for DP clustering. (Default = 1000)
--poa-cov	Specifies the maximum number of CCS reads used for POA consensus. (Default = 10)
--use-qvs	Use CCS Quality Values; sets --poa-cov to 100.
--split-bam	Splits BAM output files into a maximum of N files; 0 means no splitting. (Default = 0)
--min-subreads-split	Subread threshold for High-Quality/Low-Quality split; only works with --use-qvs. (Default = 7)
--log-level	Specifies the log level; values are [DEBUG, INFO, WARN, ERROR, CRITICAL]. (Default = WARN)
-v,--verbose	Uses verbose output.
-j,--num-threads	Specifies the number of threads to use; 0 means autodetection. (Default = 0)
--log-file	Writes the log to a file. (Default = stdout)
--emit-tool-contract	Outputs the tool contract to stdout. (Default = False)
--resolved-tool-contract	Uses arguments from the resolved tool contract.

polish Tool: Polish transcripts using subreads.

Usage

```
isoseq3 polish [options] input_1 input_2 output
```

Example

```
isoseq3 polish unpolished.bam movie.subreadset.xml polished.bam
```

Inputs/Outputs	Description
`input_1`	`unpolished.bam` file or `unpolished.transcriptset.xml` file.
`input_2`	`movie.subreads.bam` file or `movie.subreadset.xml` file.
`output`	`polished.bam` file or `polished.transcriptset.xml` file.

Options	Description
`-r,--rq-cutoff`	Specifies the RQ cutoff for `fastx` output. (Default = `0.99`)
`-c,--coverage`	Specifies the maximum number of subreads used for polishing. (Default = `60`)
`--log-level`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARN`, `ERROR`, `CRITICAL]`. (Default = `WARN`)
`-v,--verbose`	Uses verbose output.
`-j,--num-threads`	Specifies the number of threads to use; `0` means autodetection. (Default = `0`)
`--log-file`	Writes the log to a file. (Default = `stdout)`
`--emit-tool-contract`	Outputs the tool contract to `stdout`. (Default = `False`)
`--resolved-tool-contract`	Uses arguments from the resolved tool contract.

`summarize` Tool: Create a .csv-format barcode overview from transcripts.

Usage

```
isoseq3 summarize [options] input output
```

Example

```
isoseq3 summarize polished.bam summary.csv
```

Inputs/Outputs	Description
`input`	`unpolished.bam` file or `unpolished.transcriptset.xml` file.
`output`	`summary.csv` file.

Options	
`--log-level`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARN`, `ERROR`, `CRITICAL]`. (Default = `WARN`)
`-v,--verbose`	Uses verbose output.
`--log-file`	Writes the log to file. (Default = `stdout)`
`--emit-tool-contract`	Outputs the tool contract to `stdout`. (Default = `False`)
`--resolved-tool-contract`	Uses arguments from the resolved tool contract.

collapse Tool: Collapse transcripts based on genomic mapping.

Usage

```
isoseq3 collapse [options] <alignments.bam|xml> <ccs.bam|xml>
<out.fastq>
```

Examples:

```
isoseq3 collapse polished.aligned.sorted.bam out.gff
```
or
```
isoseq3 collapse polished.aligned.sorted.bam ccs.bam out.gff
```

Inputs/Outputs	Description
alignments	Alignments mapping transcripts to the reference genome. (BAM or XML file).
ccs.bam	Optional input BAM file containing CCS sequences.
out.fastq	Collapsed transcripts in FASTQ format.

Options	Description
--min-aln-coverage	Ignores alignments with less than the Minimum Query Coverage. (Default = 0.95)
--min-aln-identity	Ignores alignments with less than the Minimum Alignment Identity. (Default = 0.50)
--max-fuzzy-junction	Ignores mismatches or indels shorter than or equal to N. (Default = 5)
--version	Displays program version number and exits.
--log-file	Writes the log to file. (Default = stderr)
--log-level	Specifies the log level; values are [DEBUG, INFO, WARN, ERROR, CRITICAL]. (Default = WARN)
-j,--num-threads	Specifies the number of threads to use; 0 means autodetection. (Default = 0)

juliet

juliet is a general-purpose minor variant caller that identifies and phases minor single nucleotide substitution variants in complex populations. It identifies codon-wise variants in coding regions, performs a reference-guided *de novo* variant discovery, and annotates known drug-resistance mutations. Insertion and deletion variants are currently ignored; support will be added in a future version. There is no technical limitation with respect to the target organism or gene.

The underlying model is a statistical test, the Bonferroni-corrected Fisher's Exact test. It compares the number of observed mutated codons to the number of expected mutations at a given position.

juliet uses JSON target configuration files to define different genes in longer reference sequences, such as overlapping open reading frames in HIV. These predefined configurations ease batch applications and allow immediate reproducibility. A target configuration may contain multiple coding regions within one reference sequence and optional drug resistance mutation positions.

Notes:

- The preinstalled target configurations are meant for a quick start. It is the user's responsibility to ensure that the target configurations used are correct and up-to-date.
- If the target configuration `none` was specified, the provided reference is assumed to be in-frame.

Performance

At a coverage of 6,000 CCS reads with a predicted accuracy (RQ) of `≥0.99`, the false positive and false negative rates are below 1% and 0.001% (10^{-5}), respectively.

Usage

```
$ juliet --config "HIV" data.align.bam patientZero.html
```

Required	Description
`input_file.bam`	Input aligned BAM file containing CCS records, which must be PacBio-compliant, that is, `cigar M` is forbidden.
`output_file.html`	Output report HTML file.

Configuration	Description
`--config,-c`	Path to the target configuration JSON file, predefined target configuration tag, or the JSON string.
`--mode-phasing,-p`	Phase variants and cluster haplotypes.

Restrictions	Description
`--region,-r`	Specifies the genomic region of interest; reads are clipped to that region. Empty means **all** reads.
`--drm-only,-k`	Only reports DRM positions specified in the target configuration. Can be used to filter for drug-resistance mutations - only known variants from the target configuration are called.
`--min-perc,-m`	Specifies the minimum variant percentage to report. Example: `--min-perc 1` will only show variant calls with an observed abundance of more than 1%. (Default = `0`)
`--max-perc,-n`	Specifies the maximum variant percentage to report. Example: `--max-perc 95` will only show variant calls with an observed abundance of less than 95%. (Default = `100`)

Chemistry Override (Specify both)	Description
--sub,-s	Specifies the substitution rate. Use to override the learned rate. (Default = 0)
--del,-d	Specifies the deletion rate. Use to override the learned rate. (Default = 0)

Options	Description
--help, -h	Displays help information and exits.
--verbose, -v	Sets the verbosity level.
--version	Displays program version number and exits.
--debug	Returns all amino acids, irrespective of their relevance.
--emit-tool-contract	Emits the tool contract.
--resolved-tool-contract	Uses arguments from the resolved tool contract.
--mode-phasing,-p	Phases variants and cluster haplotypes.

Input Files

- BAM-format files containing CCS records. These must be PacBio-compliant, that is, `cigar M` is forbidden.
- Input CCS reads should have a minimal predicted accuracy of `0.99`.
- Reads should be created with CCS2 using the `--richQVs` option. Without the `--richQVs` information, the number of false positive calls might be higher, as `juliet` is missing information to filter actual heteroduplexes in the sample provided.
- `juliet` currently does **not** demultiplex barcoded data; you must provide one BAM file per barcode.

Output Files

A JSON and/or HTML file:

```
$ juliet data.align.bam patientZero.html
$ juliet data.align.bam patientZero.json
$ juliet data.align.bam patientZero.html patientZero.json
```

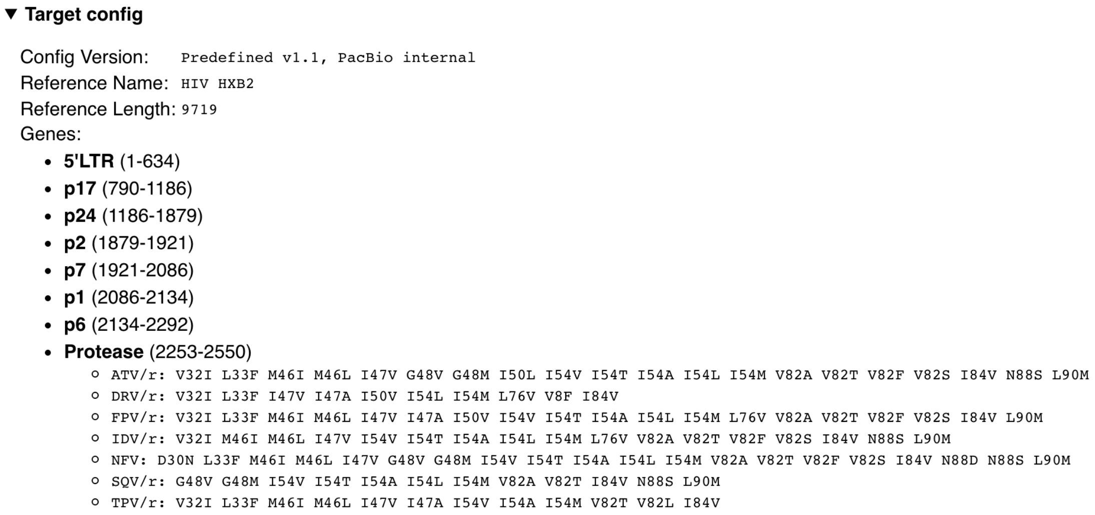
The HTML file includes the same content as the JSON file, but in more human-readable format. The HTML file contains four sections:

1. Input Data

Summarizes the data provided, the exact call for `juliet`, and `juliet` version for traceability purposes.

2. Target Config

Summarizes details of the provided target configuration for traceability. This includes the configuration version, reference name and length, and annotated genes. Each gene name (in bold) is followed by the reference start, end positions, and possibly known drug resistance mutations.



▼ **Target config**

Config Version: `Predefined v1.1, PacBio internal`
Reference Name: `HIV HXB2`
Reference Length: `9719`
Genes:

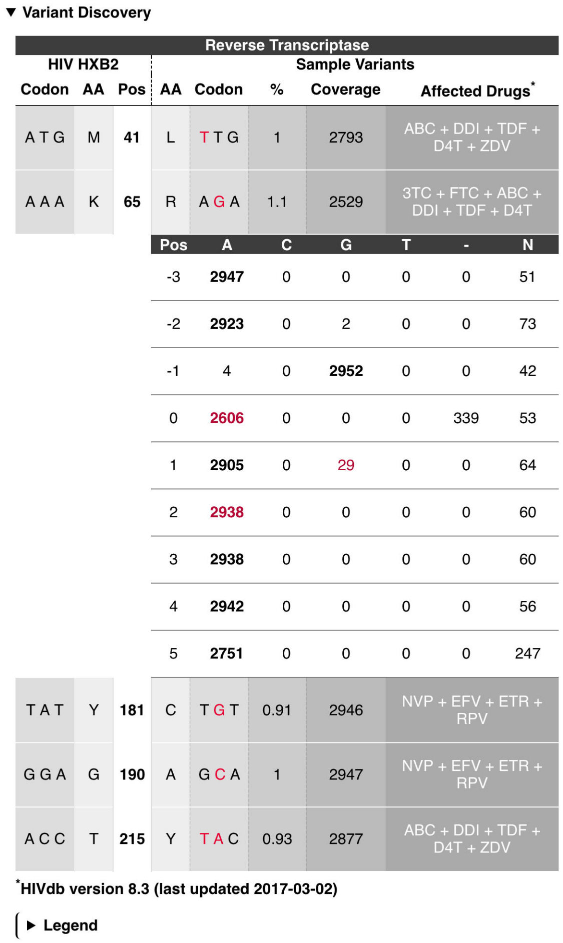
- **5'LTR** (1-634)
- **p17** (790-1186)
- **p24** (1186-1879)
- **p2** (1879-1921)
- **p7** (1921-2086)
- **p1** (2086-2134)
- **p6** (2134-2292)
- **Protease** (2253-2550)
  - `ATV/r: V32I L33F M46I M46L I47V G48V G48M I50L I54V I54T I54A I54L I54M V82A V82T V82F V82S I84V N88S L90M`
  - `DRV/r: V32I L33F I47V I47A I50V I54L I54M L76V V8F I84V`
  - `FPV/r: V32I L33F M46I M46L I47V I47A I50V I54V I54T I54A I54L I54M L76V V82A V82T V82F V82S I84V L90M`
  - `IDV/r: V32I M46I M46L I47V I54V I54T I54A I54L I54M L76V V82A V82T V82F V82S I84V N88S L90M`
  - `NFV: D30N L33F M46I M46L I47V G48V G48M I54V I54T I54A I54L I54M V82A V82T V82F V82S I84V N88D N88S L90M`
  - `SQV/r: G48V G48M I54V I54T I54A I54L I54M V82A V82T I84V N88S L90M`
  - `TPV/r: V32I L33F M46I M46L I47V I47A I54V I54A I54M V82T V82L I84V`

3. Variant Discovery

For each gene/open reading frame, there is one overview table.

Each row represents a variant position.

- Each variant position consists of the reference codon, reference amino acid, relative amino acid position in the gene, mutated codon, percentage, mutated amino acid, coverage, and possible affected drugs.
- Clicking the row displays counts of the multiple-sequence alignment counts of the -3 to +3 context positions.



▼ Variant Discovery

Reverse Transcriptase							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
ATG	M	41	L	TTG	1	2793	ABC + DDI + TDF + D4T + ZDV
AAA	K	65	R	AGA	1.1	2529	3TC + FTC + ABC + DDI + TDF + D4T

Pos	A	C	G	T	-	N
-3	**2947**	0	0	0	0	51
-2	**2923**	0	2	0	0	73
-1	4	0	**2952**	0	0	42
0	**2606**	0	0	0	339	53
1	**2905**	0	29	0	0	64
2	**2938**	0	0	0	0	60
3	**2938**	0	0	0	0	60
4	**2942**	0	0	0	0	56
5	**2751**	0	0	0	0	247

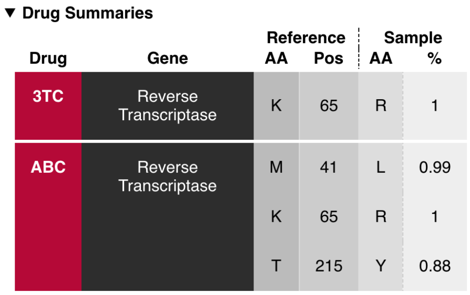
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
TAT	Y	181	C	TGT	0.91	2946	NVP + EFV + ETR + RPV
GGA	G	190	A	GCA	1	2947	NVP + EFV + ETR + RPV
ACC	T	215	Y	TAC	0.93	2877	ABC + DDI + TDF + D4T + ZDV

*HIVdb version 8.3 (last updated 2017-03-02)

▶ Legend

4. Drug Summaries

Summarizes the variants grouped by annotated drug mutations:



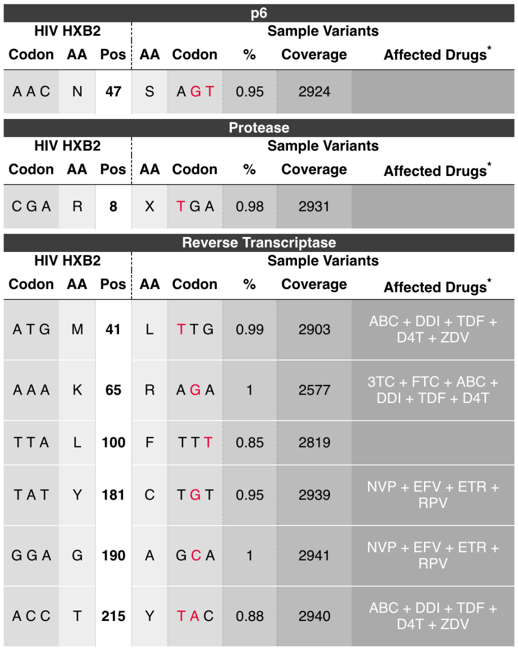
▼ Drug Summaries

		Reference		Sample	
Drug	Gene	AA	Pos	AA	%
3TC	Reverse Transcriptase	K	65	R	1
ABC	Reverse Transcriptase	M	41	L	0.99
		K	65	R	1
		T	215	Y	0.88

Predefined Target Configuration

`juliet` ships with one predefined target configuration, for HIV. Following is the command syntax for running that predefined target configuration:

```
$ juliet --config "HIV" data.align.bam patientZero.html
```



p6							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
AAC	N	47	S	AGT	0.95	2924	

Protease							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
CGA	R	8	X	TGA	0.98	2931	

Reverse Transcriptase							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
ATG	M	41	L	TTG	0.99	2903	ABC + DDI + TDF + D4T + ZDV
AAA	K	65	R	AGA	1	2577	3TC + FTC + ABC + DDI + TDF + D4T
TTA	L	100	F	TTT	0.85	2819	
TAT	Y	181	C	TGT	0.95	2939	NVP + EFV + ETR + RPV
GGA	G	190	A	GCA	1	2941	NVP + EFV + ETR + RPV
ACC	T	215	Y	TAC	0.88	2940	ABC + DDI + TDF + D4T + ZDV

- **Note**: For the predefined configuration `HIV`, please use the HIV HXB2 complete genome for alignment.

Customized Target Configuration

To define your own target configuration, create a JSON file. The root child `genes` contains a list of coding regions, with `begin` and `end`, the name of the gene, and a list of drug resistant mutations. Each DRM consists of its name and the positions it targets. The `drms` field is optional. If provided, the `referenceSequence` is used to call mutations, otherwise it will be tested against the major codon. All indices are with respect to the provided alignment space, 1-based, begin-inclusive and end-exclusive `[)`.

Target Configuration Example 1- A customized `json` target configuration file named `my_customized_hiv.json`:

```
{
    "genes": [
        {
            "begin": 2550,
            "drms": [
                {
                    "name": "fancy drug",
                    "positions": [ "M41L" ]
                }
            ],
            "end": 2700,
            "name": "Reverse Transcriptase"
        }
    ],
    "referenceName": "my seq",
    "referenceSequence": "TGGAAGGGCT...",
    "version": "Free text to version your config files"
    "databaseVersion": "DrugDB version x.y.z (last updated YYYY-MM-DD)"
}
```

Run with a customized target configuration using the `--config` option:

```
$ juliet --config my_customized_hiv.json data.align.bam patientZero.html
```

Valid Formats for DRMs/positions

`103`	**Only** the reference position.
`M130`	Reference amino acid and reference position.
`M103L`	Reference aa, reference position, mutated aa.
`M103LKA`	Reference aa, reference position, list of possible mutated aas.
`103L`	Reference position and mutated aa.
`103LG`	Reference position and list mutated aas.

Missing amino acids are processed as wildcard (`*`).

Example:

```
{ "name": "ATV/r", "positions": [ "V32I", "L33", "46IL",
"I54VTALM", "V82ATFS", "84" ] }
```

Target Configuration Example 2 - BCR-ABL:

For BCR-ABL, using the ABL1 gene with the following reference NM_005157.5 (https://www.ncbi.nlm.nih.gov/nuccore/NM_005157.5) a typical target configuration looks like this:

```
{
    "genes": [
        {
            "name": "ABL1",
            "begin": 193,
            "end": 3585,
            "drms": [
                {
                    "name": "imatinib",
                    "positions": [
                      "T315AI","Y253H","E255KV","V299L","F317AICLV","F359CIV" ]
                },
                {
                    "name": "dasatinib",
                    "positions": [ "T315AI","V299L","F317AICLV" ]
                },
                {
                    "name": "nilotinib",
                    "positions": [ "T315AI","Y253H","E255KV","F359CIV" ]
                },
                {
                    "name": "bosutinib",
                    "positions": [ "T315AI" ]
                }
            ]
        }
    ],
  "referenceName": "NM_005157.5",
    "referenceSequence": "TTAACAGGCGCGTCCC..."
```

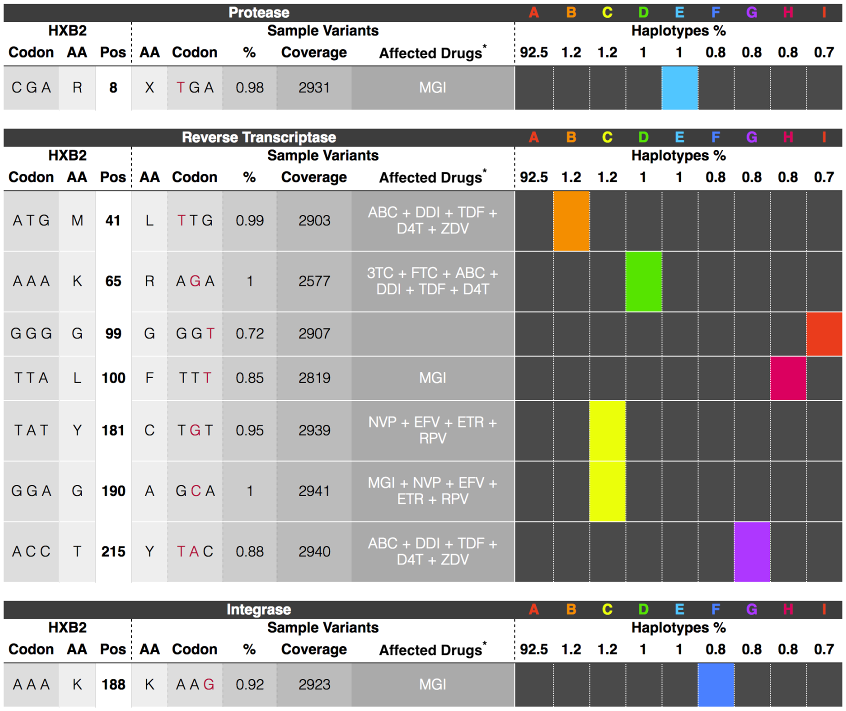
No Target Configuration

If **no** target configuration is specified, either make sure that the sequence is in-frame, or specify the region of interest to mark the correct reading frame, so that amino acids are correctly translated. The output is labeled with `unknown` as the gene name:

```
$ juliet data.align.bam patientZero.html
```

Phasing

The default mode is to call amino-acid/codon variants independently. Using the `--mode-phasing` option, variant calls from distinct haplotypes are clustered and visualized in the HTML output.



Protease								A	B	C	D	E	F	G	H	I
HXB2			Sample Variants					Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
CGA	R	8	X	TGA	0.98	2931	MGI					■				

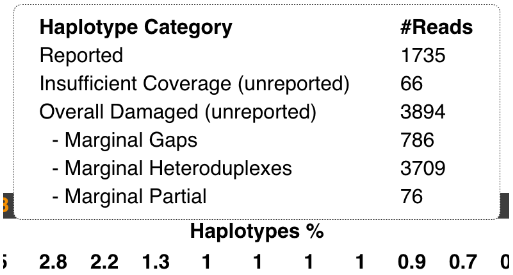
Reverse Transcriptase								A	B	C	D	E	F	G	H	I
HXB2			Sample Variants					Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
ATG	M	41	L	TTG	0.99	2903	ABC + DDI + TDF + D4T + ZDV		■							
AAA	K	65	R	AGA	1	2577	3TC + FTC + ABC + DDI + TDF + D4T				■					
GGG	G	99	G	GGT	0.72	2907										■
TTA	L	100	F	TTT	0.85	2819	MGI								■	
TAT	Y	181	C	TGT	0.95	2939	NVP + EFV + ETR + RPV			■						
GGA	G	190	A	GCA	1	2941	MGI + NVP + EFV + ETR + RPV			■						
ACC	T	215	Y	TAC	0.88	2940	ABC + DDI + TDF + D4T + ZDV							■		

Integrase								A	B	C	D	E	F	G	H	I
HXB2			Sample Variants					Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
AAA	K	188	K	AAG	0.92	2923	MGI						■			

- The row-wise variant calls are "transposed" onto per-column haplotypes. Each haplotype has an ID: `[A-Z]{1}[a-z]?`.
- For each variant, colored boxes in this row mark haplotypes that contain this variant.
- Colored boxes per haplotype/column indicate variants that co-occur. Wild type (no variant) is represented by plain dark gray. A color palette helps to distinguish between columns.
- The JSON variant positions has an additional `haplotype_hit` boolean array with the length equal to the number of haplotypes. Each entry indicates if that variant is present in the haplotype. A haplotype block under the root of the JSON file contains counts and read names. The order of those haplotypes matches the order of all `haplotype_hit` arrays.

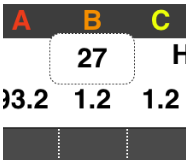
There are two types of tooltips in the haplotype section of the table.

The first tooltip is for the **Haplotypes %** and shows the number of reads that count towards (A) Actually reported haplotypes, (B) Haplotypes that have less than 10 reads and are not being reported, and (C) Haplotypes that are not suitable for phasing. Those first three categories are mutually exclusive and their sum is the total number of reads going into `juliet`. For (C), the three different marginals provide insights into the sample quality; as they are marginals, they are not exclusive and can overlap. The following image shows a sample with bad PCR conditions:



Haplotype Category	#Reads
Reported	1735
Insufficient Coverage (unreported)	66
Overall Damaged (unreported)	3894
- Marginal Gaps	786
- Marginal Heteroduplexes	3709
- Marginal Partial	76

The second type of tooltip is for each haplotype percentage and shows the number of reads contributing to this haplotype:



laa Long Amplicon Analysis (LAA) finds phased consensus sequences from a pooled set of (possibly polyploid) amplicons sequenced with Pacific Biosciences' SMRT technology. Sometimes referred to as **LAA2**, the executable `laa` is a complete rewrite of the `AmpliconAnalysis` module from the `ConsensusTools` package included with earlier versions of SMRT Analysis, which performed a similar function in the Quiver framework. `laa` is a computational and memory-intensive software tool that builds upon the Arrow framework for generating high-quality consensus sequences. It is generally preferable to run `laa` using the SMRT Link interface for efficient distribution across a compute cluster. However, it is occasionally useful to run `laa` from the command-line to identify optimal parameter settings or to diagnose a problem.

Run Modes

`AmpliconAnalysis` is a general solution for the analysis of PCR products generated with SMRT sequencing, and it can be run in multiple configurations depending on the design of the experiment.

1. **Pooled Polyploid Amplicons**: The default mode assumes that the data contains a single complex mixture of amplicons, which may come from different genes and may have multiple alleles.
2. **Barcoded Polyploid Amplicons:** If passed a file of barcoding results, `AmpliconAnalysis` will instead separate the data by barcode and run the above process on each subset.
3. **Barcoded Simple Amplicons:** Another common use case is to generate consensus sequences for a large number of simple amplicons, such as for synthetic construct validation or high-throughput screening.

Input Files

`laa` **only** accepts PacBio-compatible BAM files or Data Set XML files as input.

In addition, the underlying files themselves now contain barcode information. This document assumes that you already have a barcoded PacBio BAM file containing the data to be analyzed.

Output Files

`laa` produces two sets of FASTQ files containing a sequence for each phased template sequence in each coarse cluster, and for each barcode.

- `amplicon_analysis.fastq`: Contains all of the high-quality non-artifactual sequences found.
- `amplicon_analysis_chimeras_noise.fastq`: Contains sequences thought to be some form of PCR or sequencing artifact.

  Note: A sequence is defined as an artifact if, in the summary CSV file, the value of either the `IsDuplicate`, `NoiseSequence` or `IsChimera` column is `True`.
- `amplicon_analysis_summary.csv`: Contains summary information about each read. Empty fields and values of `-1` represent inapplicable columns, while fields with `1` represent `True` and `0` represents `False`. Contains the following fields:
  - `BarcodeName`: Name of the barcode the reads came from. This is set to `0` for non-barcode runs.
  - `FastaName`: Sequence ID or header string.
  - `CoarseCluster`: Number of the coarse cluster the sequence came from.
  - `Phase`: Number of the phase of the sequence in the coarse cluster.
  - `TotalCoverage`: Total number of subreads mapped to this sequence. This may be capped using the `numPhasingReads` option.
  - `SequenceLength`: Length of this consensus sequence.
  - `ConsensusConverged`: `1` if a final consensus was reached within the allotted iterations; `0` if otherwise. `0` may indicate problems with the underlying sample or data.

- PredictedAccuracy: Predicted accuracy of the consensus sequence, calculated by multiplying together the QVs generated by Arrow.
  - NoiseSequence: 1 if the sequence has a low-quality consensus, corresponding to a predicted accuracy less than 95% indicating a probable PCR artifact; 0 if otherwise.
  - IsDuplicate: 1 if the sequence is a duplicate of another with more coverage; 0 if otherwise.
  - DuplicateOf: If IsDuplicate is 1, contains the name of the other sequence; otherwise empty.
  - IsChimera: 1 if the sequence is tagged as a chimeric by the UCHIME-like chimera labeler; 0 if otherwise.
  - ChimeraScore: UCHIME-like score for sequences tested as possible chimeras.
  - ParentSequenceA: If chimeric, the name of the consensus thought to be the source of the left half.
  - ParentSequenceB: If chimeric, the name of the consensus thought to be the source of the right half.
  - CrossoverPosition: Position in the chimeric sequence where the junction between the parent sequences is thought to have occurred.
- amplicon_analysis_subreads.X.csv: Contains mapping probabilities for each subread used to call the consensus sequences generated. A **separate** file is written for **each** barcode pair, where X is replaced with the name of that pair. Contains the following fields:
  - SubreadId: The name of a particular subread used in the current run.
  - <A Consensus Sequence Name>: The mapping probability for the subread listed in SubreadId to the particular consensus sequence named.

Usage

```
laa [options] INPUT
```

Options	Description
-h, --help	Displays help information and exits.
--verbose, -v	Sets the verbosity level.
--version	Displays program version number and exits.
--log level	Sets the logging level. (Default = INFO)
--rngSeed	RNG seed, modulates reservoir filtering of reads. (Default = 42)
--generateBamIndex	Generates PacBio indicies (*.pbi) for BAM files that don't have them.
--ignoreBamIndex	Ignores PacBio indicies (*.pbi) for BAM files if they exist.
-M,--modelPath	Specifies the path to a model file or directory containing model files.
-m,--modelSpec	Specifies the name of chemistry or model to use, overriding the default selection.
--numThreads,-n	Specifies the number of threads to use; 0 means autodetection. (Default = 0)

Options	Description
`--takeN`	Reports only the top `N` consensus sequences for each barcode. To **disable**, use a number less than 1. (Default = `0`)
`-t,--trimEnds`	Trims `N` bases from each end of each consensus. (Default = `0`)
`--minPredictedAccuracy`	Specifies the minimum predicted consensus accuracy below which a consensus is treated as noise. (Default = `0.949999988079071`)
`--chimeraScoreThreshold`	Specifies the minimum score to consider a sequence chimeric. (Default = `1`)
`--ChimeraFilter`	Activates the chimera filter and separate chimeric consensus outputs.
`--noChimeraFilter`	Deactivates the chimera filter and outputs all consensus.
`--logFile`	Output file to write logging information to.
`--resultFile`	Output file name for high-quality results. (Default = `amplicon_analysis.fastq`)
`--junkFile`	Output file name for low-quality or chimeric results. (Default = `amplicon_analysis_chimeras_noise.fastq`)
`--reportFile`	Output file name for the summary report. (Default = `amplicon_analysis_summary.csv`)
`--inputReportFile`	Output file name for the output estimates of input PCR quality, based on subread mappings. (Default = `amplicon_analysis_input.csv`)
`--subreadsReportPrefix`	Prefix for the output subreads report. (Default = `amplicon_analysis_subreads`)
`-b,--barcodes`	Specifies the FASTA file name of the barcode sequences used, which **overwrites** any barcode names in the Data Set. **Note**: This is used **only** to find barcode names.
`--minBarcodeScore`	Specifies the minimum average barcode score required for subreads. (Default = `0`)
`--fullLength`	Filters input reads by presence of both flanking barcodes.
`--doBc`	Specifies a comma-separated list of barcode pairs to analyse. This can be by name ("`lbc1--lbc1`") or by Index ("`0--0`").
`--ignoreBc`	Disables barcode filtering so that all data be treated as one sample.
`-l,--minLength`	Specifies the minimum length of input reads to use. (Default = `3000`)
`-L,--maxLength`	Specifies the maximum length of input reads to use. To **disable**, set to `0`. (Default = `0`)
`-s,--minReadScore`	Specifies the minimum read score of input reads to use. (Default = `0.75`)
`--minSnr`	Specifies the minimum SNR of input reads to use. (Default = `3.75`)
`--whitelist`	Specifies a file of ReadIds, in either Text or FASTA format, to allow from the input file. (Default = `NONE`)
`-r,--maxReads`	Specifies the maximum number of input reads, per barcode, to use in analysis. (Default = `2000`)
`-c,--maxClusteringReads`	Specifies the maximum number of input reads to use in the initial clustering step. (Default = `500`)
`--fullLengthPreference`	Prefers full-length subreads when clustering.
`--fullLengthClustering`	Uses only full-length subreads when clustering.
`--clusterInflation`	Markov clustering inflation parameter. (Default = `2`)

Options	Description
`--clusterLoopWeight`	Markov clustering loop weight parameter. (Default = `0.00100000004749745`)
`--skipRate`	Skips some high-scoring alignments to disperse the cluster more. (Default = `0.0`)
`--minClusterSize`	Specifies the minimum number of reads supporting a cluster before it is reported. (Default = `20`)
`--doCluster`	Only analyzes one specified cluster. (Default = `-1`)
`--Clustering`	Enables coarse clustering.
`--noClustering`	Disables coarse clustering.
`-i,--ignoreEnds`	When splitting, ignores `N` bases at the end. This prevents excessive splitting caused by degenerate primers. (Default = `0`)
`--maxPhasingReads`	Specifies the maximum number of reads to use for phasing/consensus. (Default = `500`)
`--minQScore`	Specifies the minimum score to require of mutations used for phasing. (Default = `20`)
`--minPrevalence`	Specifies the minimum prevalence to require of mutations used for phasing. (Default = `0.0900000035762787`)
`--minSplitReads`	Specifies the minimum number of reads favoring the minor phase required to split a haplotype. (Default = `20`)
`--minSplitFraction`	Specifies the minimum fraction of reads favoring the minor phase required to split a haplotype. (Default = `0.100000001490116`)
`--minSplitScore`	Specifies the global likelyhood improvement required to split a haplotype. (Default = `500`)
`--minZScore`	Specifies the minimum Z Score to allow before adding a read to a haplotype. (Default = `-10`)
`--Phasing`	Enables the fine phasing step.
`--noPhasing`	Disables the fine phasing step.
`--emit-tool-contract`	Emits the tool contract.
`--resolved-tool-contract`	Uses arguments from the resolved tool contract.

Algorithm Description

`laa` proceeds in six main phases: Data filtering, coarse clustering, waterfall clustering, fine phasing, consensus polishing, and post-processing.

- **Data filtering** is used to separate out sequences by their barcode calls, if present, so that only reads long enough to meaningfully contribute to phasing are used.
- The **Coarse and Waterfall Clustering** steps are used to find and separate reads coming from different amplicons.
- The reads from each cluster are then put through the **phasing** step, which recursively separates full-length haplotypes using a variant of the Arrow model. Those haplotypes are then **polished** within the Arrow framework to achieve a high-quality consensus sequence.

- Finally, a **post-processing** step attempts to identify and remove spurious consensus sequences and sequences representing PCR artifacts.

Data Filtering

In this first step, we separate sequences by barcode and then apply a series of user-selected quality filters to speed up down-stream processing and improve result quality. Filters are used primarily to remove short subreads (which may not be long enough to phase variants of interest) and subreads with low barcode scores (representing reads for whom the barcode call is uncertain and may be incorrect). A “Whitelist” option is also available so that users can specify the exact list of subreads or ZMWs to use.

Coarse Clustering and Waterfall Clustering

The coarse clustering step groups the number of subreads (set by the `maxClusteringReads` option) that originate from different amplicons into different clusters. It works by detecting subread-to-subread similarities, building a graph of the results, and then clustering nodes (subreads) using the Markov Clustering algorithm (http://micans.org/mcl/). The Markov clustering step is needed to remove spurious similarities caused by chimeric reads that can arise from PCR errors or doubly-loaded ZMWs, or just by chance due to sequencing error.

Next, if the number of subreads specified with the `maxReads` option is greater than the number used in coarse clustering, any remaining subreads are aligned to a rough consensus of each cluster and added to the cluster with the greatest similarity. This “waterfall” step allows for a larger number of reads to be used much more quickly than if all subreads had to be clustered using the normal coarse clustering process.

At the end of clustering, subreads in each cluster are then sorted for downstream analysis using the PageRank algorithm (Page, Lawrence, et al. “The PageRank citation ranking: Bringing order to the web.” (1999)). This ensures that the most representative reads of the cluster are used first in the generation of consensus sequences.

Phasing/Consensus

The reads assigned to each cluster are loaded into the Arrow framework, and an initial consensus of all reads is found. SNP differences between subreads and the initial consensus are scored with the Arrow model, and combinations of high-scoring SNPs are tested for their ability to segregate the reads into multiple haplotypes. If sufficient evidence of a second haplotype is found, the template sequence is “split” into two copies, one with the SNPs applied to the template and one without. This process is repeated recursively so long as new haplotypes with sufficient scores can be found with at least some minimum level of coverage.

Post-Processing Filters

`laa` implements a post-processing step to flag likely PCR artifacts in the set of phased output sequences. First, consensus sequences that are identical duplicates of other consensus sequences in the results are removed. Next, those with unusually low predicted accuracy are flagged as being probable sequencing artifacts and removed. PacBio implemented a filter for consensus sequences from PCR crossover events, which on average make up ~5 to 20% of products generated by PCR amplifications >3 kb in length.

For artifacts of PCR crossover events, or “chimeras”, PacBio implemented a variant of the UCHIME algorithm (Edgar, Robert C., et al. “UCHIME improves sensitivity and speed of chimera detection.” Bioinformatics 27.16(2011): 2194-2200). The consensus sequences are sorted in order of decreasing read coverage, and the first two sequences are accepted as non-chimeric since they have no possible parent sequences with greater coverage. The remaining sequences are evaluated in descending order, with **each** test sequence aligned to all non-chimeric sequences so far processed. Crossovers between pairs of non-chimeric sequences are checked to see if they would yield a sequence very similar to the test sequence. If one is found with a sufficient score, the test sequence is marked as chimeric. If not, the test sequence is added to the list of non-chimeric sequences.

motifMaker

The `motifMaker` tool identifies motifs associated with DNA modifications in prokaryotic genomes. Modified DNA in prokaryotes commonly arises from restriction-modification systems that methylate a specific base in a specific sequence motif. The canonical example is the m6A methylation of adenine in GATC contexts in *E. coli*. Prokaryotes may have a very large number of active restriction-modification systems present, leading to a complicated mixture of sequence motifs.

PacBio SMRT sequencing is sensitive to the presence of methylated DNA at single base resolution, via shifts in the polymerase kinetics observed in the real-time sequencing traces. For more background on modification detection, see http://nar.oxfordjournals.org/content/early/2011/12/07/nar.gkr1146.full**.**

Algorithm

Existing motif-finding algorithms such as MEME-chip and YMF are sub-optimal for this case for the following reasons:

- They search for a **single** motif, rather than attempting to identify a complicated mixture of motifs.
- They generally don't accept the notion of aligned motifs - the input to the tools is a window into the reference sequence which can contain the motif at any offset, rather than a single center position that is available with kinetic modification detection.

- Implementations generally either use a Markov model of the reference (MEME-chip), or do exact counting on the reference, but place restrictions on the size and complexity of the motifs that can be discovered.

Following is a rough overview of the algorithm used by `motifMaker`: Define a motif as a set of tuples: (position relative to methylation, required base). Positions not listed in the motif are implicitly degenerate. Given a list of modification detections and a genome sequence, define the following objective function on motifs:

```
Motif score(motif) = (# of detections matching motif) / (# of genome sites matching
motif) * (Sum of log-pvalue of detections matching motif) = (fraction methylated) * (sum
of log-pvalues of matches)
```

Then, search (close to exhaustively) through the space of all possible motifs, progressively testing longer motifs using a branch-and-bound search. The “fraction methylated” term must be less than 1, so the maximum achievable score of a child node is the sum of scores of modification hits in the current node, allowing pruning of all search paths whose maximum achievable score is less than the best score discovered so far.

Usage

Run the `find` command, and pass the reference FASTA and the `modifications.gff` (.gz) file output by the PacBio modification detection workflow.

The `reprocess` subcommand annotates the GFF file with motif information for better genome browsing.

```
MotifMaker [options] [command] [command options]
```

`find` Command: Run motif-finding.

```
find [options]
```

Options	Description
-h, --help	Displays help information and exits.
* -f, --fasta	Reference FASTA file.
* -g, --gff	`Modifications.gff` or `.gff.gz` file.
-m, --minScore	Specifies the minimum Qmod score to use in motif finding. (Default = `40.0`)
* -o, --output	Outputs `motifs.csv` file.
-x, --xml	Outputs motifs XML file.

`reprocess` Command: Update a `modifications.gff` file with motif information based on new Modification QV thresholds.

```
reprocess [options]
```

Options	Description
`-c, --csv`	Raw `modifications.csv` file.
`* -f, --fasta`	Reference FASTA file.
`* -g, --gff`	`Modifications.gff` or `.gff.gz` file.
`-m, --minFraction`	Specifies that only motifs above this methylated fraction are used. (Default = `0.75`)
`-m, --motifs`	`Motifs.csv` file.
`* -o, --output`	Reprocessed `modifications.gff` file.

Output Files

Using the `find` command:

- **Output CSV file**: This file has the same format as the standard "Fields included in motif_summary.csv" described in the Methylome Analysis White Paper (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Methylome-Analysis-Technical-Note).

Using the `reprocess` command:

- **Output GFF file**: The format of the output file is the same as the input file, and is described in the Methylome Analysis White Paper under "Fields included in the modifications.gff file" (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Methylome-Analysis-Technical-Note).

pbalign

The `pbalign` tool aligns PacBio reads to reference sequences; filters aligned reads according to user-specified filtering criteria; and converts the output to PacBio BAM, SAM, or PacBio Data Set format.

Input Files

The `pbalign` tool distinguishes input and output file formats by file extensions. The tool supports the following input formats:

- BAM: `.bam`
- Data Set: `.subreadset.xml` or `.consensusreadset.xml`
- FASTA: `.fa` or `.fasta`
- File-Of-File-Names: `.fofn`

The input reference sequences can be in a FASTA file or a reference Data Set.

Output Files

The tool supports the following output formats:

- BAM: `.bam`
- Data Set: `.xml`
- SAM: `.sam`

Usage

```
pbalign [-h] [--verbose] [--version] [--profile] [--debug]
        [--regionTable REGIONTABLE] [--configFile CONFIGFILE]
        [--algorithm {blasr,bowtie}] [--maxHits MAXHITS]
        [--minAnchorSize MINANCHORSIZE]
        [--maxMatch MAXMATCH]
        [--useccs {useccs,useccsall,useccsdenovo}]
        [--noSplitSubreads] [--nproc NPROC]
        [--algorithmOptions ALGORITHMOPTIONS]
        [--maxDivergence MAXDIVERGENCE] [--minAccuracy MINACCURACY]
        [--minLength MINLENGTH]
        [--scoreFunction {alignerscore,editdist,blasrscore}]
        [--scoreCutoff SCORECUTOFF]
        [--concordant]
        [--hitPolicy {randombest,allbest,random,all}] [--forQuiver]
        [--seed SEED] [--tmpDir TMPDIR]
        inputFileName referencePath outputFileName
```

Required	Description
`inputFileName`	The input file of PacBio reads. Can be a BAM, Data Set, FASTA file, or a `fofn` (File-Of-File-Names).
`referencePath`	Either a reference FASTA file or a PacBio reference Data Set file.
`outputFileName`	The output `.bam, .xml` or `.sam` file.

Options	Description
`-h, --help`	Displays help information and exits.
`--verbose, -v`	Sets the verbosity level.
`--version`	Displays program version number and exits.
`--profile`	Prints runtime profile at exit.
`--debug`	Runs within a debugger session.
`--configFile`	Specifies a set of user-defined argument values.
`--algorithm`	Selects an algorithm from `blasr` or `bowtie`. (Default = `blasr`)
`--maxHits`	Specifies the maximum number of matches of each read to the reference sequence to evaluate. (Default = `10`)
`--minAnchorSize`	Specifies the length of the read that must match against the reference sequence. (Default = `12`)
`--maxMatch`	Stops extending an anchor between the read and the reference sequence when its length reaches this value. Bypasses the blasr `maxMatch` option. (Default = `30`)

Options	Description
`--noSplitSubreads`	Does **not** split reads into subreads even if subread regions are available. (Default = `False`)
`--concordant`	Maps subreads of a ZMW to the same genomic location. (Default = `False`)
`--nproc NPROC`	Specifies the number of threads. (Default = `8`)
`--algorithmOptions`	Passes alignment options through. **Note**: By default, `blasr` places gap inconsistently when aligning a sequence and its reverse complement sequence. It is preferable to place gap **consistently** to call a consensus sequence from multiple alignments or call single nucleotide variants (SNPs), as the output alignments will make it easier for variant callers to call variants. To do so, specify `--algorithmOptions=' --placeGapConsistently'.`
`--maxDivergence`	Specifies the maximum allowed percentage divergence of a read from the reference sequence. (Default = `30`)
`--minAccuracy`	Specifies the minimum percentage accuracy of alignments to evaluate. (Default = `70`)
`--minLength`	Specifies the minimum aligned read length of alignments to evaluate. (Default = `50`)
`--scoreFunction`	Specifies a score function for evaluating alignments. • `alignerscore`: Aligner's score in the SAM tag `as`. • `editdist`: Edit distance between read and reference. • `blasrscore`: The `blasr` default score function. (Default = `alignerscore`)
`--scoreCutoff`	Specifies the worst score to output an alignment.
`--hitPolicy`	Specifies a policy for how to treat multiple hits. • `random`: Selects a random hit. • `all`: Selects **all** hits. • `allbest`: Selects all the best score hits. • `randombest`: Selects a random hit from all best alignment score hits. • `leftmost`: Reports an alignment which has the best alignment score and has the smallest mapping coordinates in any reference. (Default = `randombest`)
`--seed`	Initializes the random number generator with a non-zero integer. `0` means that current system time is used. (Default = `1`)
`--tmpDir`	Specifies a directory for saving temporary files. (Default = `/scratch`)

Examples

Basic usage:

```
$ pbalign tests/data/example/read.bam   \
          tests/data/example/ref.fasta  \
          tests/data/example/example.bam
```

Basic usage with optional arguments:

```
$ pbalign --maxHits 10 --hitPolicy all  \
            tests/data/example_read.fasta \
            tests/data/example_ref.fasta  \
            example.sam
```

Advanced usage - To import predefined options from a configuration file:

```
$ pbalign --configFile=tests/data/1.config \
            tests/data/example/read.fasta    \
            tests/data/example/ref.fasta     \
            example.sam
```

Advanced usage - To pass options through to the Aligner:

```
$ pbalign --algorithmOptions='-nCandidates 10 -sdpTupleSize 12 --placeGapConsistently'\
            tests/data/example/read.fasta \
            tests/data/example/ref.fasta   \
            example.sam                   \
```

Advanced usage - To use `pbalign` as a library using the Python API:

```
$ python
>>> from pbalign.pbalignrunner import PBAlignRunner
>>> # Specify arguments in a list.
>>> args = ['--maxHits', '20', 'tests/data/example/read.fasta',\
...         'tests/data/example/ref.fasta', 'example.sam']
>>> # Create a PBAlignRunner object.
>>> a = PBAlignRunner(args)
>>> # Execute.
>>> exitCode = a.start()
>>> # Show all files used.
>>> print a.fileNames
```

pbcromwell

The `pbcromwell` tool is Pacific Biosciences' wrapper for the `cromwell` scientific workflow engine used to power SMRT Link. `pbcromwell` includes advanced utilities for interacting directly with a Cromwell server.

`pbcromwell` is designed primarily for running workflows distributed and supported by PacBio, but it is written to handle any valid WDL source (version 1.0), and is very flexible in how it takes input. PacBio workflows are expected to be found in the directory defined by the `SMRT_PIPELINE_BUNDLE_DIR` environment variable, which is automatically defined by the SMRT Link distribution.

Note that `pbcromwell` does **not** interact with SMRT Link services; to run a `Cromwell` workflow as a SMRT Link job, please use `pbservice`. (For details, see "pbservice" on page 71.)

Note: Interaction with the `Cromwell` server is primarily intended for developers and power users.

Usage

```
pbcromwell {run,show-workflows,show-workflow-details,configure,submit,get-job,abort,metadata,show-running,wait}
```

Enter `pbcromwell {command} -h` for a command's options.

Examples:

Show available PacBio-developed workflows:

```
  $ pbcromwell --quiet show-workflows
```

Show details for a PacBio workflow:

```
  $ pbcromwell --quiet show-workflow-details pb_ccs
```

Generate `cromwell.conf` with HPC settings:

```
  $ pbcromwell configure --default-backend SGE --output-file cromwell.conf
```

Launch a PacBio workflow:

```
  $ pbcromwell run pb_ccs -e /path/to/movie.subreadset.xml --nproc 8 --config /full/
path/to/cromwell.conf
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.
`--log-file LOG_FILE`	Writes the log to file. (Default = `None,` writes to `stdout.`)
`--log-level=INFO`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARNING`, `ERROR`, `CRITICAL.]` (Default = `INFO`)
`--debug`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet`	Alias for setting the log level to `CRITICAL` to suppress output. (Default = `False`)
`--verbose, -v`	Sets the verbosity level. (Default = `None`)

`pbcromwell run` Command: Run a Cromwell workflow. Multiple input modes are supported, including a `pbsmrtpipe`-like set of arguments (for PacBio workflows **only**), and JSON files already in the native Cromwell format.

Usage:

```
pbcromwell run [-h] [--output-dir OUTPUT_DIR] [--overwrite] [-i INPUTS]
                    [-e ENTRY_POINTS] [-n NPROC] [-c MAX_NCHUNKS]
                    [--target-size TARGET_SIZE] [--queue QUEUE] [-o OPTIONS]
                    [-t TASK_OPTIONS] [-b BACKEND] [-r MAX_RETRIES]
                    [--tmp-dir TMP_DIR] [--config CONFIG] [--dry-run]
                    workflow
```

Options	Description
`--output-dir OUTPUT_DIR`	Output directory for Cromwell output. (Default = `cromwell_out`)

Options	Description
`--overwrite`	Overwrites the output directory, if it exists. (Default = `False`)
`-i INPUTS, --inputs INPUTS`	Cromwell inputs and settings as JSON files. (Default = `None`)
`-e ENTRY_POINTS, --entry ENTRY_POINTS`	Entry point Data Set; may be repeated for workflows that take more than one input Data Set. Note that **all** PacBio workflows require at least **one** such entry point.
`-n NPROC, --nproc NPROC`	Number of processors per task. (Default = `1`)
`-c MAX_NCHUNKS, --max-nchunks MAX_NCHUNKS`	Maximum number of chunks per task. (Default = `None`)
`--target-size TARGET_SIZE`	Target chunk size. (Default = `None`)
`--queue QUEUE`	Cluster queue to use. (Default = `None`)
`-o OPTIONS, --options OPTIONS`	Additional Cromwell engine options, as a JSON file. (Default = `None`)
`-t TASK_OPTIONS, --task-option TASK_OPTIONS`	Workflow- or task-level option as `key=value` strings, specific to the application. May be specified multiple times for multiple options. (Default = `[]`)
`-b BACKEND, --backend BACKEND`	Backend to use for running tasks. (Default = `None`)
`-r MAX_RETRIES, --maxRetries MAX_RETRIES`	Maximum number of times to retry a failing task. (Default = `1`)
`--tmp-dir TMP_DIR`	Optional temporary directory for Cromwell tasks, which **must** exist on all compute hosts. (Default = `None`)
`--config CONFIG`	Java configuration file for running Cromwell. (Default = `None`)
`--dry-run`	Don't execute Cromwell, just write final inputs and then exit. (Default = `True`)
`workflow`	Workflow ID (such as `pb_ccs` or `cromwell.workflows.pb_ccs` for PacBio workflows only) or q path to a Workflow Description Language (WDL) source file.

All PacBio workflows have similar requirements to the `pbsmrtpipe` pipelines in previous SMRT Link versions:

1. One or more PacBio dataset XML entry points, usually a SubreadSet or ConsensusReadSet (`--entry-point <FILE>`.)
2. Any number of workflow-specific task options `(--task-option <OPTION>`.)
3. Engine options independent of the workflow, such as number of processors per task (`--nproc`), or compute backend (`--backend`).

Output is directed to a new directory: `--output-dir`, which defaults to `cromwell_out`. This includes final inputs for the Cromwell CLI, and subdirectories for logs (workflow and task outputs), links to output files, and the Cromwell execution itself, which has a complex nested directory structure. Detailed information about the workflow execution can be found in the file `metadata.json`, in the native Cromwell format.

Note that output file links do **not** include the individual resource files of datasets and reports (BAM files, index files, plot PNGs, and so on.) Follow the symbolic links to their real path (for example using `readlink -f`) to find report plots.

For further information about Cromwell, consult the official documentation at https://cromwell.readthedocs.io.

Workflow Examples:

Run the CCS workflow:

```
$ pbcromwell run pb_ccs -e <SUBREADS> --nproc 8 --config /full/path/to/cromwell.conf
```

Run the Iso-Seq workflow, including mapping to a reference, and execute on SGE:

```
$ pbcromwell run pb_isoseq3 -e <SUBREADS> -e <PRIMERS> -e <REFERENCE> --nproc 8 --
config /full/path/to/cromwell.conf
```

Run a user-defined workflow:

```
$ pbcromwell run my_workflow.wdl -i inputs.json -o options.json --config /full/path/to/
cromwell.conf
```

Set up input files but exit before starting Cromwell:

```
$ pbcromwell run pb_ccs -e <SUBREADS> --nproc 8 --dry-run
```

Print details about the named PacBio workflow, including input files and task options. **Note**: The prefix `cromwell.workflows.` is optional.

```
$ pbcromwell show-workflow-details pb_ccs
$ pbcromwell show-workflow-details cromwell.workflows.pb_ccs
```

pbdagcon

The `pbdagcon` tool implements DAGCon (Directed Acyclic Graph Consensus), which is a sequence consensus algorithm based on using directed acyclic graphs to encode multiple sequence alignments.

`pbdagcon` uses the alignment information from `blasr` to align sequence reads to a "backbone" sequence. Based on the underlying alignment directed acyclic graph (DAG), it uses the new information from the reads to find the discrepancies between the reads and the "backbone" sequences. A dynamic programming process is then applied to the DAG to find the optimum sequence of bases as the consensus. The new consensus can be used as a new backbone sequence to iteratively improve the consensus quality.

While the code is developed for processing Pacific Biosciences raw sequence data, the algorithm can be used for general consensus purposes. Currently, it only takes FASTA input. For shorter read sequences, one might need to adjust the `blasr` alignment parameters to get the alignment string properly.

Note: This code is **not** an official Pacific Biosciences software release.

Examples

To generate consensus from blasr alignments:

This is the most basic use case to generate a consensus from a set of alignments by directly using the pbdagcon executable.

At the most basic level, pbdagcon takes information from blasr alignments sorted by target and generates FASTA-formatted corrected target sequences. The alignments from blasr can be formatted with either -m 4 or -m 5. For -m 4 format, the alignments **must** be run through a format adapter (m4topre.py) to generate suitable input to pbdagcon.

The following example shows the simplest way to generate a consensus for one target using blasr -m 5 alignments as input:

```
blasr queries.fasta target.fasta -bestn 1 -m 5 -out mapped.m5
pbdagcon mapped.m5 > consensus.fasta
```

To generate corrected reads from daligner alignments:

Support for generating consensus from daligner output exists as a new executable: dazcon. Note that dazcon is sensitive to the version of daligner used and may fail if using inputs generated by versions other than what is referenced in the submodules.

```
dazcon -ox -j 4 -s subreads.db -a subreads.las > corrected.fasta
```

To correct PacBio reads using HGAP:

This example shows how PacBio reads are corrected in PacBio's "Hierarchical Genome Assembly Process" (HGAP) workflow. HGAP uses blasr -m 4 output.

This example makes use of the filterm4.py and m4topre.py scripts:

```
# First filter the m4 file to help remove chimeras:
filterm4.py mapped.m4 > mapped.m4.filt

# Next run the m4 adapter script, generating 'pre-alignments':
m4topre.py mapped.m4.filt mapped.m4.filt reads.fasta 24 > mapped.pre

# Finally, correct using pbdagcon, typically using multiple consensus threads:
pbdagcon -j 4 -a mapped.pre > corrected.fasta
```

pbindex The pbindex tool creates an index file that enables random access to PacBio-specific data in BAM files.

Usage

```
pbindex <input>
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.

Input File

- `*.bam` file containing PacBio data.

Output File

- `*.pbi` index file, with the same prefix as the input file name.

pbmm2

The `pbmm2` tool aligns native PacBio data, outputs PacBio BAM files, and is a SMRT `minimap2` wrapper for PacBio data.

Note: `pbmm2` is the official replacement for `blasr` and `pbalign`.

`pbmm2` supports native PacBio input and output, provides sets of recommended parameters, generates sorted output on-the-fly, and post-processes alignments. Sorted output can be used directly for polishing using `GenomicConsensus`, if BAM has been used as input to `pbmm2`.

Benchmarks show that `pbmm2` runs faster than `blasr` and outperforms it in mapped concordance and number of mapped bases.

`pbmm2` adds the following SAM tags to each aligned record:

- `mc`, stores mapped concordance percentage between 0.0 and 100.0.
- `rm`, is set to 1 if alignment has been manipulated by repeated matches trimming.

Usage

```
pbmm2 <tool>
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.

`index` Command: Indexes references and stores them as `.mmi` files. Indexing is optional, but recommended if you use the same reference with the same `--preset` multiple times.

Usage:

```
pbmm2 index [options] <ref.fa|xml> <out.mmi>
```

Input File

- `*.fasta`, `*.fa` file containing reference contigs or `*.referenceset.xml`.

Output File

- `out.mmi` (minimap2 index file.)

Notes:

- You can use existing `minimap2` .mmi files with `pbmm2 align`.
- If you use an index file, you **cannot** override parameters `-k`, `-w`, nor `-u` in `pbmm2 align`.
- The `minimap2` parameter `-H` (homopolymer-compressed k-mer) is always on for SUBREAD and UNROLLED presets, and can be disabled using `-u`.

Options	Description
`--preset`	Specifies the alignment mode: • `"SUBREAD" -k 19 -w 10` • `"CCS" -k 19 -w 10 -u` • `"ISOSEQ" -k 15 -w 5 -u` • `"UNROLLED" -k 15 -w 15` (Default = `SUBREAD`)
`-k`	Specifies the k-mer size, which cannot be larger than `28`. (Default = `-1`)
`-w`	Specifies the Minimizer window size. (Default = `-1`)
`-u,--no-kmer-compression`	Disables homopolymer-compressed k-mer. (Compression is on by default for the SUBREAD and UNROLLED presets.)

`align` Command: Aligns PacBio reads to reference sequences. The output argument is optional; if not provided, the BAM output is streamed to `stdout`.

Usage:

```
pbmm2 align [options] <ref.fa|xml|mmi> <in.bam|xml|fa|fq> [out.aligned.bam|xml]
```

Input Files

- `*.fasta` file containing reference contigs, or `*.referenceset.xml`, or `*.mmi` index file.
- `*.bam`, `*.subreadset.xml`, `*.consensusreadset.xml`, `*.transcriptset.xml`, `*.fasta`, `*.fa`, `*.fastq`, or `*.fastq` file containing PacBio data.

Output Files

- `*.bam` aligned reads in BAM format.
- `*.alignmentset`, `*.consensusalignmentset.xml`, or `*.transcriptalignmentset.xml` if XML output was chosen.

The following Data Set Input/output combinations are allowed:

SubreadSet > AlignmentSet

```
pbmm2 align hg38.referenceset.xml movie.subreadset.xml hg38.movie.alignmentset.xml
```

ConsensusReadSet > ConsensusAlignmentSet

```
pbmm2 align hg38.referenceset.xml movie.consensusreadset.xml
hg38.movie.consensusalignmentset.xml --preset CCS
```

TranscriptSet > TranscriptAlignmentSet

```
pbmm2 align hg38.referenceset.xml movie.transcriptset.xml
hg38.movie.transcriptalignmentset.xml --preset ISOSEQ
```

FASTA/Q input

In addition to native PacBio BAM input, reads can also be provided in FASTA and FASTQ formats.

Attention: The resulting output BAM file **cannot** be used as input into `GenomicConsensus`!

With FASTA/Q input, the `--rg` option sets the read group. Example:

```
pbmm2 align hg38.fasta movie.Q20.fastq hg38.movie.bam --preset CCS --rg
'@RG\tID:myid\tSM:mysample'
```

All three reference file formats `.fasta`, `.referenceset.xml`, and `.mmi` can be combined with FASTA/Q input.

Options	Description
`-h, --help`	Displays help information and exits.
`--chunk-size`	Processes `N` records per chunk. (Default = `100`)
`--sort`	Generates a sorted BAM file.
`-m,--sort-memory`	Specifies the memory per thread for sorting. (Default = `768M`)
`-j,--alignment-threads`	Specifies the number of threads used for alignment. `0` means autodetection. (Default = `0`)
`-J,--sort-threads`	Specifies the number of threads used for sorting. `0` means 25% of `-j`, with a maximum of `8`. (Default = `0`)
`--sample`	Specifies the sample name for all read groups. Defaults, in order of precedence: A) SM field in the input read group B) Biosample name C) Well sample name D) "`UnnamedSample`".
`--rg`	Specifies the read group header line such as `'@RG\tID:xyz\tSM:abc'`. Only for FASTA/Q inputs.
`-c,--min-concordance-perc`	Specifies the minimum alignment concordance, in percent. (Default = `70`)
`-l,--min-length`	Specifies the minimum mapped read length, in base pairs. (Default = `50`)
`-N,--best-n`	Specifies the output at maximum `N` alignments for each read. `0` means no maximum. (Default = `0`)
`--strip`	Removes all kinetic and extra QV tags. The output cannot be polished.

Options	Description
`--split-by-sample`	Specifies one output BAM file per sample.
`--no-bai`	Omits BAI index file generation for sorted output.
`--unmapped`	Specifies that unmapped records be included in the output.
`--median-filter`	Picks one read per ZMW of median length.
`--zmw`	Processes ZMW Reads; `subreadset.xml` input is required. This activates the UNROLLED preset.
`--hqregion`	Processes the HQ region of each ZMW; `subreadset.xml` input is required. This activates the UNROLLED preset.

Parameter Set Options and Overrides	Description
`--preset`	Specifies the alignment mode: • `"SUBREAD" -k 19 -w 10 -o 5 -O 56 -e 4 -E 1 -A 2 -B 5 -z 400 -Z 50 -r 2000 -L 0.5` • `"CCS" -k 19 -w 10 -u -o 5 -O 56 -e 4 -E 1 -A 2 -B 5 -z 400 -Z 50 -r 2000 -L 0.5` • `"ISOSEQ" -k 15 -w 5 -u -o 2 -O 32 -e 1 -E 0 -A 1 -B 2 -z 200 -Z 100 -C 5 -r 200000 -G 200000 -L 0.5` • `"UNROLLED" -k 15 -w 15 -o 2 -O 32 -e 1 -E 0 -A 1 -B 2 -z 200 -Z 100 -r 2000 -L 0.5` (Default = `SUBREAD`)
`-k`	Specifies the k-mer size, which cannot be no larger than `28`. (Default = `-1`)
`-w`	Specifies the Minimizer window size. (Default = `-1`)
`-u,--no-kmer-compression`	Disables homopolymer-compressed k-mer. (Compression is on by default for the SUBREAD and UNROLLED presets.)
`-A`	Specifies the matching score. (Default = `-1`)
`-B`	Specifies the mismatch penalty. (Default = `-1`)
`-z`	Specifies the Z-drop score. (Default = `-1`)
`-Z`	Specifies the Z-drop inversion score. (Default = `-1`)
`-r`	Specifies the bandwidth used in chaining and DP-based alignment. (Default = `-1`)
`-o,--gap-open-1`	Specifies the gap open penalty 1. (Default = `-1`)
`-O,--gap-open-2`	Specifies the gap open penalty 2. (Default = `-1`)
`-e,--gap-extend-1`	Specifies the gap extension penalty 1. (Default = `-1`)
`-E,--gap-extend-2`	Specifies the gap extension penalty 2. (Default = `-1`)
`-L,--lj-min-ratio`	Specifies the long join flank ratio. (Default = `-1`)
`-G`	Specifies the maximum intron length; this changes `-r`. (Default = `-1`)
`-C`	Specifies the cost for a non-canonical GT-AG splicing. (Default = `-1`)
`--no-splice-flank`	Specifies that you do **not** prefer splicing flanks GT-AG.

Examples:

Generate an index file for reference and reuse it to align reads:

```
$ pbmm2 index ref.fasta ref.mmi
$ pbmm2 align ref.mmi movie.subreads.bam ref.movie.bam
```

Align reads and sort on-the-fly, with 4 alignment and 2 sort threads:

```
$ pbmm2 align ref.fasta movie.subreads.bam ref.movie.bam --sort -j 4 -J 2
```

Align reads, sort on-the-fly, and create a PBI:

```
$ pbmm2 align ref.fasta movie.subreadset.xml ref.movie.alignmentset.xml --sort
```

Omit the output file and stream the BAM output to `stdout`:

```
$ pbmm2 align hg38.mmi movie1.subreadset.xml | samtools sort > hg38.movie1.sorted.bam
```

Align the CCS fastq input and sort the output:

```
$ pbmm2 align ref.fasta movie.Q20.fastq ref.movie.bam --preset CCS --sort --rg
'@RG\tID:myid\tSM:mysample'
```

Alignment Parallelization

The number of alignment threads can be specified using the `-j,--alignment-threads` option. If **not** specified, the maximum number of threads will be used, minus one thread for BAM I/O and minus the number of threads specified for sorting.

Sorting

Sorted output can be generated using the `--sort` option.

- By default, 25% of threads specified with the `-j` option (Maximum = `8`) are used for sorting.
- To override the default percentage, the `-J,--sort-threads` option defines the explicit number of threads used for on-the-fly sorting. The memory allocated per sort thread is defined using the `-m,--sort-memory` option, accepting suffixes `M`,`G`.

Benchmarks on human data show that 4 sort threads are recommended, but that no more than 8 threads can be effectively leveraged, even with 70 cores used for alignment. We recommend that you provide more memory to **each** of a **few** sort threads to avoid disk I/O pressure, rather than providing less memory to each of many sort threads.

What are parameter sets and how can I override them?

Per default, `pbmm2` uses recommended parameter sets to simplify the multitudes of possible combinations. Please see the available parameter sets in the option table shown earlier.

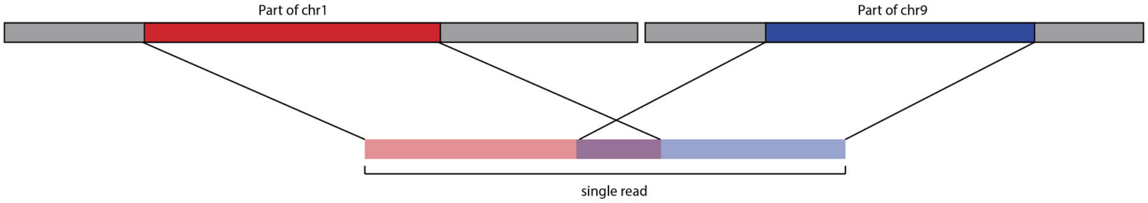
What other special parameters are used implicitly?

To achieve alignment behavior similar to `blasr`, we implicitly use the following `minimap2` parameters:

- Soft clipping with `-Y`.
- Long cigars for tag `CG` with `-L`.
- `X/=` cigars instead of `M` with `--eqx`.
- No overlapping query intervals with repeated matches trimming.
- No secondary alignments are produced using the `--secondary=no` option.

What is repeated matches trimming?

A repeated match occurs when the same query interval is shared between a primary and supplementary alignment. This can happen for translocations, where breakends share the same flanking sequence:



And sometimes, when a LINE gets inserted, the flanks are duplicated, leading to complicated alignments, where we see a split read sharing a duplication. The inserted region itself, mapping to a random other LINE in the reference genome, may also share sequence similarity to the flanks:

To get the best alignments, `minimap2` decides that two alignments may use up to 50% (default) of the same query bases. This does **not** work for PacBio, as `pbmm2` is a `blasr` replacement and requires that a single base may never be aligned twice. `Minimap2` offers a feature to enforce a query interval overlap to 0%. If a query interval gets used in two alignments, one or both get flagged as secondary and get filtered. This leads to yield loss, and more importantly, missing SVs in the alignment.

Papers (such as this) present dynamic programming approaches to find the optimal split to uniquely map query intervals, while maximizing alignment scores. We don't have per base alignment scores available, thus our approach is much simpler. We align the read, find overlapping query intervals, determine one alignment to be maximal reference-spanning, then trim all others. By trimming, `pbmm2` rewrites the cigar and the reference coordinates on-the-fly. This allows us to increase the number of mapped bases, which slightly reduces mapped concordance, but boosts SV recall rate.

How can I set the sample name?

You can override the sample name (`SM` field in the `RG` tag) for **all** read groups using the `--sample` option. If not provided, sample names derive from the Data Set input using the following order of precedence: A) SM field in the input read group B) Biosample name C) Well sample name D) UnnamedSample. If the input is a BAM file and the --sample option was **not** used, the SM field will be populated with UnnamedSample.

Can I split output by sample name?

Yes, the --split-by-sample option generates one output BAM file per sample name, with the sample name as the file name prefix, if there is more than one aligned sample name.

Can I remove all those extra per-base and per-pulse tags?

Yes, the --strip option removes the following extraneous tags if the input is BAM: dq, dt, ip, iq, mq, pa, pc, pd, pe, pg, pm, pq, pt, pv, pw, px, sf, sq, st. Note that the resulting output BAM file **cannot** be used as input into GenomicConsensus.

Where are the unmapped reads?

Per default, unmapped reads are omitted. You can add them to the output BAM file using the --unmapped option.

Can I output at maximum the N best alignments per read?

Use the option -N, --best-n. If set to 0, (the default), maximum filtering is disabled.

Is there a way to only align one subread per ZMW?

Using the --median-filter option, only the subread closest to the median subread length per ZMW is aligned. Preferably, full-length subreads flanked by adapters are chosen.

pbservice

The pbservice tool performs a variety of useful tasks within SMRT Link.

- To get help for pbservice, use pbservice -h.
- To get help for a specific pbservice command, use pbservice <command> -h.

Note: Starting in SMRT Link v6.0.0, pbservice now requires authentication when run from a remote host, using the same credentials used to log in to the SMRT Link GUI. (This also routes all requests through HTTPS port 8243, so the services port is **not** required if authentication is used.) Access to services running on localhost will continue to work without authentication.

All pbservice commands include the following optional parameters:

Options	Description
--host=http://localhost	Specifies the server host. Override the default with the environmental variable PB_SERVICE_HOST.

Options	Description
`--port=8070`	Specifies the server port. Override the default with the environmental variable `PB_SERVICE_PORT`.
`--log-file LOG_FILE`	Writes the log to file. (Default = `None,` writes to `stdout`.)
`--log-level=INFO`	Specifies the log level; values are `[DEBUG`, `INFO`, `WARNING`, `ERROR`, `CRITICAL.]` (Default = `INFO`)
`--debug=False`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet=False`	Alias for setting the log level to `CRITICAL` to suppress output. (Default = `False`)
`--user USERNAME`	Specifies the user to authenticate as; this is **required** if the target host is anything other than `localhost`.
`--ask-pass`	Prompts the user to enter a password.
`--password PASSWORD`	Supplies the password directly. This exposes the password in the shell history (or log files), so this option is **not** recommended unless you are using a limited account without Unix login access.

`status` Command: Use to get system status.

```
pbservice status [-h] [--host HOST] [--port PORT]
                 [--log-file LOG_FILE]
                 [--log-level INFO}
                 [--debug] [--quiet]
```

`import-dataset` Command: Import Local Data Set XML. The file location **must** be accessible from the host where the services are running; often on a shared file system.

```
pbservice import-dataset [-h] [--host HOST] [--port PORT]
                         [--log-file LOG_FILE]
                         [--log-level INFO]
                         [--debug] [--quiet]
                         xml_or_dir
```

Required	Description
`xml_or_dir`	Specifies a directory or XML file for the Data Set.

`import-fasta` Command: Import a FASTA file and convert to a ReferenceSet file. The file location **must** be accessible from the host where the services are running; often on a shared file system.

```
pbservice import-fasta [-h] --name NAME --organism ORGANISM --ploidy
                       PLOIDY [--block] [--host HOST] [--port PORT]
                       [--log-file LOG_FILE]
                       [--log-level INFO]
                       [--debug] [--quiet]
                       fasta_path
```

Required	Description
fasta_path	Path to the FASTA file to import.

Options	Description
--name	Specifies the name of the ReferenceSet to convert the FASTA file to.
--organism	Specifies the name of the organism.
--ploidy	Ploidy.
--block=False	Blocks during importing process.

run-analysis Command: Run a secondary analysis pipeline using an analysis.json file.

```
pbservice run-analysis [-h] [--host HOST] [--port PORT]
                       [--log-file LOG_FILE]
                       [--log-level INFO]
                       [--debug] [--quiet] [--block]
                       json_path
```

Required	Description
json_path	Path to the analysis.json file.

Options	Description
--block=False	Blocks during importing process.

emit-analysis-template Command: Output an analysis.json template to stdout that can be run using the run-analysis command.

```
pbservice emit-analysis-template [-h] [--log-file LOG_FILE]
                                 [--log-level INFO]
                                 [--debug] [--quiet]
```

get-job Command: Get a job summary by Job Id.

```
pbservice get-job [-h] [--host HOST] [--port PORT]
                  [--log-file LOG_FILE]
                  [--log-level INFO]
                  [--debug] [--quiet]
                  job_id
```

Required	Description
job_id	Job id or UUID.

get-jobs Command: Get job summaries by Job Id.

```
pbservice get-jobs [-h] [-m MAX_ITEMS] [--host HOST] [--port PORT]
                   [--log-file LOG_FILE]
                   [--log-level INFO]
```

```
                    [--debug] [--quiet]
```

Options	Description
`-m=25, --max-items=25`	Specifies the maximum number of jobs to get.

`get-dataset` Command: Get a Data Set summary by Data Set Id or UUID.

```
pbservice get-dataset [-h] [--host HOST] [--port PORT]
                      [--log-file LOG_FILE]
                      [--log-level INFO]
                      [--debug] [--quiet]
                      id_or_uuid
```

Required	Description
`id_or_uuid`	Data Set Id or UUID.

`get-datasets` Command: Get a Data Set list summary by Data Set type.

```
pbservice get-datasets [-h] [--host HOST] [--port PORT]
                       [--log-file LOG_FILE]
                       [--log-level INFO]
                       [--debug] [--quiet] [-m MAX_ITEMS]
                       [-t DATASET_TYPE]
```

Required	Description
`-t=subreads, --dataset-type=subreads`	Specifies the type of Data Set to retrieve: `subreads, alignments, references, barcodes`.

`delete-dataset` Command: Delete a specified Data Set.
Note: This is a "soft" delete - the database record is tagged as inactive so it won't display in any lists, but the files will **not** be removed.

```
pbservice delete-dataset [-h] [--host HOST] [--port PORT]
                         [--log-file LOG_FILE]
                         [--log-level INFO]
                         [--debug] [--quiet]
                         [ID]
```

Required	Description
`ID`	A valid Data Set ID, either UUID or integer ID, for the Data Set to delete.

Examples

To obtain system status, the Data Set summary, and the job summary:

```
pbservice status --host smrtlink-release --port 9091
```

To import a Data Set XML:

```
pbservice import-dataset --host smrtlink-release --port 9091 \
path/to/subreadset.xml
```

To obtain a job summary using the Job Id:

```
pbservice get-job --host smrtlink-release --port 9091 \
--log-level CRITICAL 1
```

To obtain Data Sets by using the Data Set Type `subreads`:

```
pbservice get-datasets --host smrtlink-alpha --port 8081 \
--quiet --max-items 1 -t subreads
```

To obtain Data Sets by using the Data Set Type `alignments`:

```
pbservice get-datasets --host smrtlink-alpha --port 8081 \
--quiet --max-items 1 -t alignments
```

To obtain Data Sets by using the Data Set Type `references`:

```
pbservice get-datasets --host smrtlink-alpha --port 8081 \
--quiet --max-items 1 -t references
```

To obtain Data Sets by using the Data Set Type `barcodes`:

```
pbservice get-datasets --host smrtlink-alpha --port 8081 \
--quiet --max-items 1 -t barcodes
```

To obtain Data Sets by using the Data Set UUID:

```
pbservice get-dataset --host smrtlink-alpha --port 8081 \
--quiet 43156b3a-3974-4ddb-2548-bb0ec95270ee
```

pbsv

`pbsv` is a structural variant caller for PacBio reads. It identifies structural variants and large indels (Default: ≥20 bp) in a sample or set of samples relative to a reference. `pbsv` identifies the following types of variants: Insertions, deletions, duplications, copy number variants, inversions, and translocations.

`pbsv` takes as input read alignments (BAM) and a reference genome (FASTA); it outputs structural variant calls (VCF).

Usage:

```
pbsv [-h] [--version] [--quiet] [--verbose]
          {discover,call}...
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.
`--log-file`	Logs to a file, instead of stdout.
`--log-level`	Specifies the log level; values are `[TRACE,DEBUG,INFO,WARN, FATAL.]` (Default = `WARN`)
`discover`	Finds structural variant signatures in read alignments (BAM to SVSIG).

Options	Description
`call`	Calls structural variants from SV signatures and assign genotypes (SVSIG to VCF).

pbsv discover

This command finds structural variant (SV) signatures in read alignments. The input read alignments must be sorted by chromosome position. Alignments are typically generated with `pbmm2`. The output SVSIG file contains SV signatures.

Usage:

```
pbsv discover [options] <ref.in.bam|xml> <ref.out.svsig.gz>
```

Required	Description
`ref.in.bam|xml`	Coordinate-sorted aligned reads in which to identify SV signatures.
`ref.out.svsig.gz`	Structural variant signatures output.

Options	Description
`-h, --help`	Displays help information and exits.
`-s,--sample`	Overrides sample name tag from BAM read group.
`-q,--min-mapq`	Ignores alignments with mapping quality < `N`. (Default = `20`)
`-m,--min-ref-span`	Ignores alignments with reference length < `N` bp. (Default = `100`)
`-w,--downsample-window-length`	Specifies a window in which to limit coverage, in base pairs. (Default = `10K`)
`-a,--downsample-max-alignments`	Considers up to `N` alignments in a window; `0` means disabled. (Default = `20`)
`-r,--region`	Limits discovery to this reference region: `CHR|CHR:START-END`.
`-l,--min-svsig-length`	Ignores SV signatures with length < `N` bp. (Default = `7`)
`-b,--tandem-repeats`	Specifies tandem repeat intervals for indel clustering, as an input BED file.
`-k,--max-skip-split`	Ignores alignment pairs separated by > `N` bp of a read or reference. (Default = `100`)

pbsv call

This command calls structural variants from SV signatures and assigns genotypes.

The input SVSIG file is generated using `pbsv discover`. The output is structural variants in VCF format.

Usage:

```
pbsv call [options] <ref.fa|xml> <ref.in.svsig.gz|fofn>
<ref.out.vcf>
```

Required	Description
ref.fa|xml	Reference FASTA file or ReferenceSet XML file against which to call variants.
ref.in.svsig.gz|fofn	SV signatures from one or more samples. This can be either an SV signature SVSIG file generated by pbsv discover, or a FOFN of SVSIG files.
ref.out.vcf	Variant call format (VCF) output file.

Options	Description
-h, --help	Displays help information and exits.
-j,--num-threads	Specifies the number of threads to use, 0 means autodetection. (Default = 0)
-z,--chunk-length	Processes in chunks of N reference bp. (Default = “1M”)
-t,--types	Calls these SV types: "DEL", "INS", "INV", "DUP", "BND", "CNV". (Default = “DEL,INS,INV,DUP,BND,CNV”)
-m,--min-sv-length	Ignores variants with length < N bp. (Default = 20)
--min-cnv-length	Ignore CNVs with length < N bp. (Default = 1K)
--max-inversion-gap	Does not link inverted alignments with > N bp gap or overlap with flanking alignments. (Default = 1K)
--cluster-max-length-perc-diff	Does not cluster signatures with difference in length > P%. (Default = 25)
--cluster-max-ref-pos-diff	Does not cluster signatures > N bp apart in the reference. (Default = 200)
--cluster-min-basepair-perc-id	Does not cluster signatures with base pair identity < P%. (Default = 10)
-x,--max-consensus-coverage	Limits to N reads for variant consensus. (Default = 20)
-s,--poa-scores	Scores POA alignment with triplet match,mismatch,gap. (Default = “1,-2,-2“)
--min-realign-length	Considers segments with > N length for realignment. (Default = 100)
-A, --call-min-reads-all-samples	Ignores calls supported by < N reads total across samples. (Default = 2)
-O, --call-min-reads-one-sample	Ignores calls supported by < N reads in every sample. (Default = 2)
-S, --call-min-reads-per-strand-all-samples	Ignore calls supported by < N reads per strand total across samples. (Default = 1)
-P, --call-min-read-perc-one-sample	Ignores calls supported by < P% of reads in every sample. (Default = 20)
--ccs	CCS optimized parameters: -A 1 -O 1 -S 0 -P 10.
--gt-min-reads	Specifies the minimum supporting reads to assign a sample a non-reference genotype. (Default = 1)
--annotations	Annotates variants by comparing with sequences in FASTA. (Default annotations are ALU, L1, and SVA.)

Options	Description
`--annotation-min-perc-sim`	Annotates variant if sequence similarity > `P`%. (Default = `60`)
`--min-N-in-gap`	Considers ≥ `N` consecutive "`N`" bp as a reference gap. (Default = `50`)
`--filter-near-reference-gap`	Flags variants < `N` bp from a gap as "`NearReferenceGap`". (Default = `1000`)
`--filter-near-contig-end`	Flags variants < `N` bp from a contig end as “`NearContigEnd`”. (Default = `1K`)

Following is a typical SV analysis workflow starting from subreads:

1. Align PacBio reads to a reference genome, per movie:

Subreads BAM Input:

```
pbmm2 align ref.fa movie1.subreads.bam ref.movie1.bam --sort --median-filter --sample
sample1
```

CCS BAM Input:

```
pbmm2 align ref.fa movie1.ccs.bam ref.movie1.bam --sort --preset CCS --sample sample1
```

CCS FASTQ Input:

```
pbmm2 align ref.fa movie1.Q20.fastq ref.movie1.bam --sort --preset CCS --sample sample1
--rg '@RG\tID:movie1'
```

2. Discover the signatures of structural variation, per movie or per sample:

```
pbsv discover ref.movie1.bam ref.sample1.svsig.gz
pbsv discover ref.movie2.bam ref.sample2.svsig.gz
```

3. Call structural variants and assign genotypes (all samples); for CCS input append `--ccs`:

```
pbsv call ref.fa ref.sample1.svsig.gz ref.sample2.svsig.gz
ref.var.vcf
```

Launching a Multi-Sample pbsv Analysis - Requirements

1. Merge multiple Bio Sample SMRT Cells to one Data Set with the Bio Samples specified.
   - Each SMRT Cell must have exactly **one** Bio Sample name - multiple Bio Sample names **cannot** be assigned to one SMRT Cell.
   - **Multiple** SMRT Cells can have the **same** Bio Sample name.
   - **All** of the inputs need to already have the appropriate Bio Sample records in their `CollectionMetadata`. If they don't, they are treated as a **single** sample.
2. Create a ReferenceSet from a FASTA file.
   - The ReferenceSet is often already generated and registered in SMRT Link.
   - If the ReferenceSet doesn’t exist, use the `dataset create` command to create one:

```
dataset create --type ReferenceSet --name reference_name reference.fasta
```

Launching a Multi-Sample Analysis

```
# Set subreads and ref FASTA
sample1=sample1.subreadset.xml sample2=sample2.subreadset.xml
ref=reference.fasta

pbmm2 align ${ref} ${sample1} sample1.bam --sort --median-filter --sample Sample1
pbmm2 align ${ref} ${sample2} sample2.bam --sort --median-filter --sample Sample2
samtools index sample1.bam
samtools index sample2.bam
pbindex sample1.bam
pbindex sample2.bam
pbsv discover sample1.bam sample1.svsig.gz
pbsv discover sample2.bam sample2.svsig.gz
pbsv call ${ref} sample1.svsig.gz sample2.svsig.gz out.vcf
```

out.vcf: A `pbsv` VCF output file, where columns starting from column 10 represent structural variants of Sample 1 and Sample 2:

```
#CHROM   POS ID  REF ALT QUAL    FILTER  INFO    FORMAT  Sample1  Sample2
chr01   222737  pbsv.INS.1  T   TTGGTGTTTGTTGTTTTGTTTT  .   PASS
SVTYPE=INS;END=222737;SVLEN=21;SVANN=TANDEM   GT:AD:DP  0/1:6,4:10  0/1:6,5:11
```

pbvalidate

The `pbvalidate` tool validates that files produced by PacBio software are compliant with Pacific Biosciences' own internal specifications.

Input Files

`pbvalidate` supports the following input formats:

- BAM
- FASTA
- Data Set XML

See http://pacbiofileformats.readthedocs.org/en/5.1/ for further information about each format's requirements.

Usage

```
pbvalidate [-h] [--version] [--log-file LOG_FILE]
           [--log-level {DEBUG,INFO,WARNING,ERROR,CRITICAL} | --debug | --quiet | -v]
           [-c] [--quick] [--max MAX_ERRORS]
           [--max-records MAX_RECORDS]
           [--type
{BAM,Fasta,AlignmentSet,ConsensusSet,ConsensusAlignmentSet,SubreadSet,BarcodeSet,Conti
gSet,ReferenceSet,HdfSubreadSet}]
           [--index] [--strict] [-x XUNIT_OUT] [--unaligned]
           [--unmapped] [--aligned] [--mapped]
           [--contents {SUBREAD,CCS}] [--reference REFERENCE]
           file
```

Required	Description
`file`	Input BAM, FASTA, or Data Set XML file to validate.

Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
--log-file LOG_FILE	Writes the log to file. Default (None) will write to stdout.
--log-level	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL.] (Default = CRITICAL)
--debug=False	Alias for setting the log level to DEBUG. (Default = False)
--quiet	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
--verbose, -v	Sets the verbosity level. (Default = None)
--quick	Limits validation to the first 100 records (plus file header); equivalent to --max-records=100. (Default = False)
--max MAX_ERRORS	Exits after MAX_ERRORS were recorded. (Default = None; checks the entire file.)
--max-records MAX_RECORDS	Exits after MAX_RECORDS were inspected. (Default = None; checks the entire file.)
--type	Uses the specified file type instead of guessing. [BAM,Fasta,AlignmentSet,ConsensusSet,ConsensusAlignmentSet,SubreadSet,BarcodeSet,ContigSet,ReferenceSet, HdfSubreadSet] (Default = None)
--index	Requires index files:.fai or .pbi. (Default = False)
--strict	Turns on additional validation, primarily for Data Set XML. (Default = False)

BAM Options	Description
--unaligned	Specifies that the file should contain **only** unmapped alignments. (Default = None, no requirement.)
--unmapped	Alias for --unaligned. (Default = None)
--aligned	Specifies that the file should contain **only** mapped alignments. (Default = None, no requirement.)
--mapped	Alias for --aligned. (Default = None)
--contents	Enforces the read type: [SUBREAD, CCS] (Default = None)
--reference REFERENCE	Specifies the path to an optional reference FASTA file, used for additional validation of mapped BAM records. (Default = None)

Examples

To validate a BAM file:

```
$ pbvalidate in.subreads.bam
```

To validate a FASTA file:

```
$ pbvalidate in.fasta
```

To validate a Data Set XML file:

```
$ pbvalidate in.subreadset.xml
```

To validate a BAM file and its index file (`.pbi`):

```
$ pbvalidate --index in.subreads.bam
```

To validate a BAM file and exit after 10 errors are detected:

```
$ pbvalidate --max 10 in.subreads.bam
```

To validate up to 100 records in a BAM file:

```
$ pbvalidate --max-records 100 in.subreads.bam
```

To validate up to 100 records in a BAM file (equivalent to `--max-records=100`):

```
$ pbvalidate --quick in.subreads.bam
```

To validate a BAM file, using a specified log level:

```
$ pbvalidate --log-level=INFO in.subreads.bam
```

To validate a BAM file and write log messages to a file rather than to `stdout`:

```
$ pbvalidate --log-file validation_results.log in.subreads.bam
```

sawriter

The `sawriter` tool generates a suffix array file from an input FASTA file. It is used to prebuild suffix array files for reference sequences which can later be used in resequencing workflows. `sawriter` comes with `blasr`, and is independent of `python`.

Usage

```
sawriter saOut fastaIn [fastaIn2 fastaIn3 ...] [-blt p] [-larsson] [-4bit] [-manmy]
[-kar]
   or
sawriter fastaIn  (writes to fastIn.sa)
```

Options	Description
`-blt p`	Builds a lookup table on prefixes of length `p`. This speeds up lookups considerably (more than the LCP table), but misses matches less than `p` when searching.
`-4bit`	Reads in one FASTA file as a compressed sequence file.
`-larsson`	Uses the Larsson and Sadakane method to build the array. (Default)
`-mamy`	Uses the MAnber and MYers method to build the array. This is slower than the Larsson method, and produces the same result. This is mainly for double-checking the correctness of the Larsson method.

Options	Description
-kark	Uses the Karkkainen DS3 method for building the suffix array. This is probably slower than the Larsson method, but takes only N/(sqrt 3) extra space.
-welter	Uses lightweight suffix array construction. This is a bit slower than the normal Larsson method.
-welterweight N	Uses a difference cover of size N for building the suffix array. Valid values are 7, 32, 64, 111, and 2281.

summarize Modifications

The summarizeModifications tool generates a GFF summary file (alignment_summary.gff) from the output of base modification analysis (i.e. ipdSummary) combined with the coverage summary GFF generated by resequencing pipelines. This is useful for power users running custom workflows.

Usage

```
summarizeModifications [-h] [--version] [--emit-tool-contract]
                              [--resolved-tool-contract RESOLVED_TOOL_CONTRACT]
                              [--log-file LOG_FILE]
                              [--log-level {DEBUG,INFO,WARNING,ERROR,CRITICAL} | --debug
                              | --quiet | -v]
                              modifications alignmentSummary gff_out
```

Input Files

- modifications: Base Modification GFF file.
- alignmentSummary: Alignment Summary GFF file.

Output Files

- gff_out: Coverage summary for regions (bins) spanning the reference with Base Modification results for each region.

Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
--emit-tool-contract	Outputs the tool contract to stdout. (Default = False)
--resolved-tool-contract RESOLVED_TOOL_CONTRACT	Runs the tool directly from a PacBio Resolved tool contract. (Default = None)
--log-file LOG_FILE	Writes the log to file. Default (None) will write to stdout.
--log-level	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL] (Default = INFO)
--debug	Alias for setting the log level to DEBUG. (Default = False)
--quiet	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
--verbose, -v	Sets the verbosity level. (Default = None)

Appendix A - Application Entry Points and Output Files

Assembly (HGAP 4)

Analysis Application Name: `cromwell.workflows.pb_hgap4`

Entry Point

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet
```

Key Output Files

File Name	Datastore SourceId
Coverage Summary	`pb_hgap4.coverage_gff`
Alignments	`pb_hgap4.mapped`
Polished Assembly	`pb_hgap4.consensus_fasta`
Polished Assembly	`pb_hgap4.consensus_fastq`
Draft Assembly	`pb_hgap4.ofile_a_ctg_fasta, pb_hgap4.ofile_p_ctg_fasta`

Base Modification Detection

Analysis Application Name: `cromwell.workflows.pb_basemods`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Motifs and Modifications	`pb_basemods.motifs_gff`
Motifs Summary	`pb_basemods.motifs_csv`
Full Kinetics Summary	`pb_basemods.basemods_gff`
IPD Ratios	`pb_basemods.basemods_csv`

Circular Consensus Sequencing (CCS)

Analysis Application Name: `cromwell.workflows.pb_ccs`

Entry Point

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet
```

Key Output Files

File Name	Datastore SourceId
FASTQ file	`ccs_fastq_out`
FASTA file	`ccs_fasta_out`

File Name	Datastore SourceId
BAM file	`ccs_bam_out`
Consensus Sequences	`pb_ccs.ccsxml`
CCS Statistics	`pb_ccs.report_ccs`

CCS with Mapping

Analysis Application Name: `cromwell.workflows.pb_ccs_mapping`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Coverage Summary	`pb_ccs_mapping.coverage_gff`
Alignments	`pb_ccs_mapping.mapped`
FASTQ file	`ccs_fastq_out`
FASTA file	`ccs_fasta_out`
BAM file	`ccs_bam_out`
Consensus Sequences	`pb_ccs_mapping.ccsxml`
CCS Statistics	`pb_ccs_mapping.report_ccs`
Aligned BAM	`pb_ccs_mapping.mapped_bam`
BAM Index	`pb_ccs_mapping.mapped_bam_bai`

Convert BAM to FASTX

Analysis Application Name: `cromwell.workflows.pb_bam2fastx`

Entry Point

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet
```

Key Output Files

File Name	Datastore SourceId
FASTQ file(s)	`pb_bam2fastx.fastq_zip`
FASTA file(s)	`pb_bam2fastx.fasta_zip`

Demultiplex Barcodes

Analysis Application Name: `cromwell.workflows.pb_demux_subreads`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_barcode
:name: Entry eid_barcode
:fileTypeId: PacBio.DataSet.BarcodeSet
```

Key Output Files

File Name	Datastore SourceId
Barcode Report Details	`pb_demux_subreads.summary_csv`
Demultiplexed Datasets	`Pb_demux_subreads.barcoded_reads`
Unbarcoded Reads	`Pb_demux_subreads.unbarcoded`

Demultiplex Barcodes (CCS-Only)

Analysis Application Name: `cromwell.workflows.pb_demux_ccs`

Entry Points

```
:id: eid_ccs
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_barcode
:name: Entry eid_barcode
:fileTypeId: PacBio.DataSet.BarcodeSet
```

Key Output Files

File Name	Datastore SourceId
Barcode Report Details	`pb_demux_ccs.summary_csv`
Demultiplexed Datasets	`Pb_demux_ccs.barcoded_reads`
Unbarcoded Reads	`Pb_demux_ccs.unbarcoded`

Iso-Seq Analysis

Analysis Application Name: `cromwell.workflows.pb_isoseq3`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_barcode
:name: Entry eid_barcode
:fileTypeId: PacBio.DataSet.BarcodeSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Collapsed Filtered Isoforms FASTQ	`pb_isoseq3.collapse_fastq`
Collapsed Filtered Isoforms GFF	`pb_isoseq3.collapse_gff`
Group TXT	`pb_isoseq3.collapse_group`
Abundance TXT	`pb_isoseq3.collapse_abundance`
Read Stat TXT	`pb_isoseq3.collapse_readstat`
High-Quality Transcripts	`pb_isoseq3.hq_fastq`
Low-Quality Transcripts	`pb_isoseq3.lq_fastq`
CCS FASTQ	`pb_isoseq3.ccs_fastq_zip`
Full-length CCS	`pb_isoseq3.flnc_bam`
Polished Report	`pb_isoseq3.polish_report_csv`
Cluster Report	`pb_isoseq3.report_isoseq`

Iso-Seq Analysis (CCS-Only)

Analysis Application Name: `cromwell.workflows.pb_isoseq3_ccsonly`

Entry Points

```
:id: eid_ccs
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_barcode
:name: Entry eid_barcode
:fileTypeId: PacBio.DataSet.BarcodeSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Collapsed Filtered Isoforms FASTQ	`pb_isoseq3_ccsonly.collapse_fastq`
Collapsed Filtered Isoforms GFF	`pb_isoseq3_ccsonly.collapse_gff`
Group TXT	`pb_isoseq3_ccsonly.collapse_group`
Abundance TXT	`pb_isoseq3_ccsonly.collapse_abundance`
Read Stat TXT	`pb_isoseq3_ccsonly.collapse_readstat`
High-Quality Transcripts	`pb_isoseq3_ccsonly.hq_fastq`
Low-Quality Transcripts	`pb_isoseq3_ccsonly.lq_fastq`
CCS FASTQ	`pb_isoseq3_ccsonly.ccs_fastq_zip`
Full-length CCS	`pb_isoseq3._ccsonly.flnc_bam`
Polished Report	`pb_isoseq3._ccsonly.polish_report_csv`
Cluster Report	`pb_isoseq3._ccsonly.report_isoseq`

Long Amplicon Analysis (LAA)

Analysis Application Name: `cromwell.workflows.pb_laa`

Entry Point

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet
```

Key Output Files

File Name	Datastore SourceId
Consensus Sequence Statistics CSV	`pb_laa.summary_csv`
Chimeric/Noise Consensus Sequences	`pb_laa.chimeras_fastq`
Consensus Sequences	`pb_laa.consensus_fastq`
Consensus Sequences by Barcode	`pb_laa.consensus_fastq_split`
Chimeric/Noise Consensus Sequences by Barcode	`pb_laa.chimeras_fastq_split`

Mapping

Analysis Application Name: `cromwell.workflows.pb_align_ccs`

Entry Points

```
:id: eid_ccs
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Mapped reads	`pb_align_ccs.mapped`
Coverage summary	`pb_align_ccs.coverage_gff`

Microbial Assembly

Analysis Application Name:
`cromwell.workflows.pb_assembly_microbial`

Entry Point

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet
```

Key Output Files

File Name	Datastore SourceId
Polished assembly	`pb_assembly_microbial.consensus_fasta/fastq`
Polished Contigs After oriC Rotation	`pb_assembly_microbial.assembled_fasta`
Coverage Summary	`pb_assembly_microbial.coverage_gff`
Final Assembly	`pb_assembly_microbial.ncbi_fasta`

File Name	Datastore SourceId
Mapped BAM	pb_assembly_microbial.mapped

Minor Variants Analysis

Analysis Application Name: `cromwell.workflows.pb_mv_ccs`

Entry Points

```
:id: eid_ccs
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Minor Variants HTML Reports	pb_mv_ccs.juliet_html
Per-Variant Table	pb_mv_ccs.report_csv
Alignments	pb_mv_ccs.mapped

Resequencing

Analysis Application Name: `cromwell.workflows.pb_resequencing`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Coverage and Variant Call Summary	pb_resequencing.consensus_gff
Variant Calls	pb_resequencing.variants_gff
Consensus Contigs	pb_resequencing.consensus_fastq
Variant Calls	pb_resequencing.variants_vcf
Alignments	pb_resequencing.mapped
Coverage Summary	pb_resequencing.coverage_gff
Consensus Sequences	pb_resequencing.consensus_fasta
Aligned BAM	pb_resequencing.mapped_bam
BAM Index	pb_resequencing.mapped_bam_bai

Site Acceptance Test (SAT)

Analysis Application Name: `cromwell.workflows.pb_sat`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Coverage and Variant Call Summary	`pb_sat.consensus_gff`
Variant Calls	`pb_sat.variants_gff`
Consensus Contigs	`pb_sat.consensus_fastq`
Variant Calls	`pb_sat.variants_vcf`
Alignments	`pb_sat.mapped`
Coverage Summary	`pb_sat.coverage_gff`
Consensus Sequences	`pb_sat.consensus_fasta`

Structural Variant Calling

Analysis Application Name: `cromwell.workflows.pb_sv_clr`

Entry Points

```
:id: eid_subread
:name: Entry eid_subread
:fileTypeId: PacBio.DataSet.SubreadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Structural Variants	`pb_sv_clr.variants`
Aligned reads (BioSampleName)	`pb_sv_clr.alignments_by_sample_datastore`

Structural Variant Calling (CCS-Only)

Analysis Application Name: `cromwell.workflows.pb_sv_ccs`

Entry Points

```
:id: eid_ccs
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_ref_dataset
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet
```

Key Output Files

File Name	Datastore SourceId
Structural Variants	`pb_sv_ccs.variants`
Aligned reads (BioSampleName)	`pb_sv_ccs.alignments_by_sample_datastore`

Appendix B - Third Party Command-Line Tools

Following is information on the third-party command-line tools included in the `smrtcmds/bin` subdirectory.

bamtools

- A C++ API and toolkit for reading, writing, and manipulating BAM files.
- See https://sourceforge.net/projects/bamtools/ for details.

cromwell

- Scientific workflow engine used to power SMRT Link.
- See https://cromwell.readthedocs.io/en/stable/ for details.

daligner, LAsort, LAmerge, HPC.daligner

- Finds all significant local alignments between reads.
- See https://dazzlerblog.wordpress.com/command-guides/daligner-command-reference-guide/ for details.

datander

- Finds all local self-alignment between long, noisy DNA reads.
- See https://github.com/thegenemyers/DAMASKER for details.

DB2fasta, DBdump, DBdust, DBrm, DBshow, DBsplit, DBstats, Fasta2DB

Utilities that work with Dazzler databases:

- `DB2fasta`: Converts database files to FASTA format.
- `DBdust`: Runs the DUST algorithm over the reads in the untrimmed database, producing a track that marks all intervals of low complexity sequence.
- `DBdump/DBshow`: Displays a subset of the reads in the database; selects the information to show about the reads, including any mask tracks.
- `DBrm:` Deletes all the files in a given database.
- `DBsplit`: Divides a database conceptually into a series of blocks.
- `DBstats`: Shows overview statistics for all the reads in the trimmed database.
- `Fasta2DB`: Builds an initial database, or adds to an existing database, using a list of `.fasta` files.
- See https://dazzlerblog.wordpress.com/command-guides/dazz_db-command-guide/ for details.

ipython

- An interactive shell for using the Pacific Biosciences API.
- See https://ipython.org/ for details.

python

- An object-oriented programming language.
- See https://www.python.org/ for details.

REPmask, TANmask, HPC.REPmask, HPC.TANmask

- A set of programs to soft-mask all tandem and interspersed repeats in Dazzler databases when computing overlaps.
- See https://github.com/thegenemyers/DAMASKER for details.

samtools

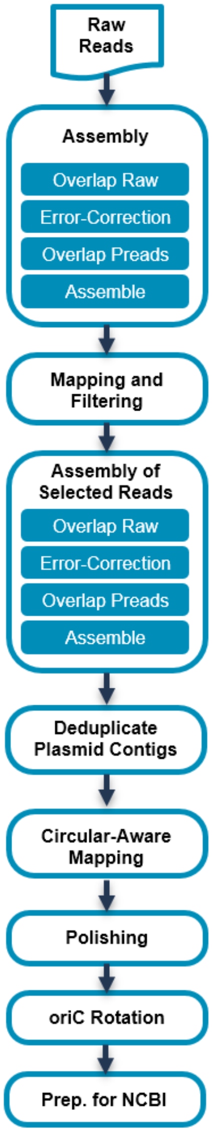
- A set of programs for interacting with high-throughput sequencing data in SAM/BAM/VCF formats.
- See http://www.htslib.org/ for details.

Appendix C - Microbial Assembly Advanced Options

Use this application to generate *de novo* assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb.

The Microbial Assembly application:

- Includes chromosomal- and plasmid-level *de novo* genome assembly, circularization, polishing, and rotation of the origin of replication for each circular contig.
- Facilitates assembly of larger genomes (yeast) as well.
- Accepts Sequel data (BAM format) as input.



The workflow shown above consists of two assembly stages:

Stage 1: Intended for contig assembly of large sequences. This stage uses the seed length cutoff which might miss small sequences in the input sample (smaller than the input cutoff, such as the plasmids).

Stage 2: Intended for a fine-grained assembly. This stage assembles only the unmapped and poorly mapped reads, does **not** use a seed length cutoff, and relaxes the overlapping parameters.

Available options for these two stages are identical. The only differences are:

1. Stage 1 parameters are prefixed with `stage1` and Stage 2 parameters with `stage2`.
2. Default values.

Complete list of all available options and their default values

```
genome_size = 5000000
coverage = 30
plasmid_contig_len_max = 300000
plasmid_min_aln_frac = 0.95
plasmid_dedup_min_frac = 0.90

stage1.length_cutoff = -1
stage1.block_size = 1024
stage1.use_median_filter = 1
stage1.ovl_opt_raw =
stage1.ovl_opt_erc =
stage1.ovl_flank_grace = 20
stage1.ovl_min_idt = 96
stage1.ovl_min_len = 1000
stage1.ovl_filter_opt = --max-diff 80 --max-cov 100 --min-cov 1 --bestn 20 --min-len
4000 --gapFilt --minDepth 4

stage2.length_cutoff = 0
stage2.block_size = 1024
stage2.use_median_filter = 1
stage2.ovl_opt_raw = --min-map-len 499
stage2.ovl_opt_erc = --min-map-len 499
stage2.ovl_flank_grace = 20
stage2.ovl_min_idt = 94
stage2.ovl_min_len = 500
stage2.ovl_filter_opt = --max-diff 10000 --max-cov 10000 --min-cov 1 --bestn 20 --min-
len 498 --gapFilt --minDepth 4
```

Advanced Parameters	Default Value	Description
`stage1.length_cutoff`	-1	Only reads as long as this value will be used as seeds in the draft assembly, and subsequently error-corrected. `-1` means this will be calculated automatically so that the total number of seed bases equals (Genome Length x Coverage). `0` means all reads in the input Data Set will be used for error-correction.
`stage1.block_size`	1024	The overlapping process is performed on pairs of blocks of input sequences, where each block contains the number of sequences which crop up to this size (in Mbp). **Note**: The number of pairwise comparisons grows quadratically with the number of blocks (meaning more cluster jobs), but also the larger the block size the more resources are required to execute each pairwise comparison.
`stage1.use_median_filter`	1	The median filter selects one subread per ZMW – the median length subread. `1` enables the filter, while `0` deactivates it. It is highly recommended to use the median filter.
`stage1.ovl_opt_raw`	NONE	Overlapping options for the `Raptor` overlapping tool, applied at the **raw read overlapping stage** (pre-assembly). The defaults are set to work well with PacBio subreads. The options set by this parameter here are fed directly into the `Raptor` call. For details on `Raptor` options, use `raptor -h.`
`stage1.ovl_opt_erc`	NONE	Overlapping options for the `Raptor` overlapping tool, applied at the **pread overlapping stage**. The defaults are set to work well with error-corrected reads and HiFi reads. The options set by this parameter here are fed directly into the `Raptor` call. For details on `Raptor` options, use `raptor -h.`
`stage1.ovl_flank_grace`	20	Heuristic to salvage some potential dovetail overlaps. Only dovetail overlaps are used for assembly, and all other overlaps (partial overlaps, which are actually **local alignments** by definition) are not used to construct the string graph. Dovetail overlaps are overlaps where the full suffix of one read and a full prefix of the other read are used to form the overlap. More details can be found here: http://wgs-assembler.sourceforge.net/wiki/index.php/Overlaps Overlaps are formed in the process of alignments, and alignment extension near the ends of the sequences can be stopped in case there are errors present near the edges of one or both of the sequences. For any overlap which is missing only a few bases to become a dovetail overlap (the number of bases defined by this parameter), the coordinates are augmented to convert it into a dovetail overlap. The impact of this parameter is very low, and this value is set to work in almost all cases. This value should also be set relatively low, to avoid chimeric overlaps.
`stage1.ovl_min_idt`	96	Overlap identity threshold (in percentage) for filtering overlaps used for contig construction.
`stage1.ovl_min_len`	1000	Minimum span of an overlap to keep it for contig construction, in bp.

Advanced Parameters	Default Value	Description
`stage1.ovl_filter_opt`	`--max-diff 80 --max-cov 100 --min-cov 1 --bestn 20 --min-len 4000 --gapFilt --minDepth 4`	Overlap filter options. These are identical to FALCON overlap filtering options except for the addition of the two options listed in the defaults: `--gapFilt` - Enables the chimera filter, which analyzes each pread's overlap pile, and determines whether a pread is chimeric based on the local coverage across the pread. `--minDepth` - Option for the chimera filter. The chimera filter is ignored when a local region of a read has coverage lower than this value. The other parameters are: `--min-cov` - Minimum allowed coverage at either the 5' or the 3' end of a read. If the coverage is below this value, the read is blacklisted and all of the overlaps it is incident with are ignored. This helps remove potentially chimeric reads. `--max-cov` - Maximum allowed coverage at either the 5' or the 3' end of a read. If the coverage is above this value, the read is blacklisted and all of the overlaps it is incident with are ignored. This helps remove repetitive reads which can make hairballs in the string graph. Note that this value is a heuristic which works well for ~30x seed length cutoff. If the cutoff is set higher, it is advised that this value is also increased. `--max-diff` - Maximum allowed difference between the coverages at the 5' and 3' ends of any particular read. If the coverage is above this value, the read is blacklisted and all of the overlaps it is incident with are ignored. `--bestn` - Keep at most this many overlaps on the 5' and the 3' side of any particular read. `--min-len` - Filter overlaps where either A-read or the B-read are shorter than this value.
`stage2.length_cutoff`	0	Only reads as long as this value will be used as seeds in the draft assembly, and subsequently error-corrected. `-1` means this will be calculated automatically so that the total number of seed bases equals (Genome Length x Coverage). `0` means all reads in the input Data Set will be used for error-correction.
`stage2.block_size`	1024	The overlapping process is performed on pairs of blocks of input sequences, where each block contains the amount of sequences which crop up to this size (in Mbp). **Note**: The number of pairwise comparisons grows quadratically with the number of blocks (meaning: more cluster jobs), but also the larger the block size the more resources are required to execute each pairwise comparison.
`stage2.use_median_filter`	1	The median filter selects one subread per ZMW – the median length subread. `1` enables the filter, while `0` deactivates it. It is highly recommended to use the median filter.
`stage2.ovl_opt_raw`	`--min-map-len 499`	Overlapping options for the `Raptor` overlapping tool, applied at the **raw read overlapping stage** (pre-assembly). The defaults are set to work well with PacBio subreads. The options set by this parameter here are fed directly into the `Raptor` call. For details on `Raptor` options, use `raptor -h.` The option `--min-map-len` reduces the minimum span of the overlap to 499 bp (instead of the default 1000 bp). This allows shorter overlaps to be reported.

Advanced Parameters	Default Value	Description
`stage2.ovl_opt_erc`	`--min-map-len 499`	Overlapping options for the `Raptor` overlapping tool, applied at the **pread overlapping stage**. The defaults are set to work well with error-corrected reads and HiFi reads. The options set by this parameter here are fed directly into the `Raptor` call. For details on `Raptor` options, use `raptor -h`. The option `--min-map-len` reduces the minimum span of the overlap to 499 bp (instead of the default 1000 bp). This allows shorter overlaps to be reported.
`stage2.ovl_flank_grace`	20	Heuristic to salvage some potential dovetail overlaps. Only dovetail overlaps are used for assembly, and all other overlaps (partial overlaps, which are actually **local alignments** by definition) are not used to construct the string graph. Dovetail overlaps are overlaps where the full suffix of one read and a full prefix of the other read are used to form the overlap. More details can be found here: http://wgs-assembler.sourceforge.net/wiki/index.php/Overlaps Overlaps are formed in the process of alignments, and alignment extension near the ends of the sequences can be stopped in case there are errors present near the edges of one or both of the sequences. For any overlap which is missing only a few bases to become a dovetail overlap (the number of bases defined by this parameter), the coordinates are augmented to convert it into a dovetail overlap. The impact of this parameter is very low, and this value is set to work in almost all cases. This value should also be set relatively low, to avoid chimeric overlaps.
`stage2.ovl_min_idt`	94	Overlap identity threshold (in percentage) for filtering overlaps used for contig construction.
`stage2.ovl_min_len`	500	Minimum span of an overlap to keep it for contig construction, in bp.
`genome_size`	5,000,000	The approximate number of base pairs expected in the genome, used to determine the coverage cutoff. **Note**: It is better to slightly overestimate rather than underestimate the genome length to ensure good coverage across the genome.
`Coverage`	30	A target value for the total amount of subread coverage used for assembly. This parameter is used, together with the genome size, to calculate the seed length cutoff.
`plasmid_contig_len_max`	300,000	Maximum expected plasmid size in the input subreadset. The default value covers a large range of possible plasmids. This value is used to select subreads for the secondary assembly stage which is specialized for assembly of smaller sequences (e.g. plasmids) that might have been lost due to the seed length cutoff threshold. Any contig assembled in the first assembly stage larger than this value will be filtered out and reassembled in the secondary assembly stage. This is performed in order to avoid partially assembled plasmid sequences
`plasmid_min_aln_frac`	0.95	Applied in the "Mapping and filtering" stage, where raw subreads are aligned to the filtered contigs of the first assembly stage. Any subread which doesn't have at least this large of aligned span (in query coordinates) is kept for the secondary assembly stage, in addition to all reads which didn't align) The value is a fraction of the subread's length (`0.95` means 95% of the subread's size).

Advanced Parameters	Default Value	Description
`plasmid_dedup_min_frac`	0.90	Applied in the "Deduplicate plasmid contigs" stage, where contigs from the secondary assembly stage are aligned to the contigs of the first assembly stage. This is done because reusing unmapped and poorly mapped reads can still cause duplicate contigs to form in the secondary assembly stage. After contigs from the secondary stage are aligned, any contig whose alignment doesn't cover at least this fraction of it's length is kept. All other contigs are marked as duplicates and removed.

P/N 100-939-900 Version 09 (November 2019)