SMRT® Tools Reference Guide

Introduction

This document describes the command-line tools included with SMRT® Link v10.2. 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 (v10.2)**.

- 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
```

Supported Chemistry

SMRT Link v10.2 supports all chemistry versions for **Sequel® II Systems** and chemistry v2.1 and later for **Sequel Systems**.

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 list of hole numbers, or a percentage of reads to be randomly selected. See "bamsieve" on page 3.
`ccs`	Calculates consensus sequences from multiple "passes" around a circularized single DNA molecule (SMRTbell® template). See "ccs" on page 6.
`dataset`	Creates, opens, manipulates and writes Data Set XML files. See "dataset" on page 14.
`Demultiplex Barcodes`	Identifies barcode sequences in PacBio single-molecule sequencing data. See "Demultiplex Barcodes" on page 20.

Tool	Description
`export-datasets`	Takes one or more PacBio dataset XML files and packages all contents into a single ZIP archive. See “export-datasets” on page 32.
`export-job`	Takes one SMRT Link Analysis job and packages all contents into a single ZIP archive. See“export-job” on page 33.
`gcpp`	Variant-calling tool which provides several variant-calling algorithms for PacBio sequencing data. See “gcpp” on page 34.
`Genome Assembly`	Generates *de novo* assemblies using HiFi Reads. See “Genome Assembly” on page 36.
`HiFiViral SARS-CoV-2 Analysis`	Analyzes multiplexed viral surveillance samples for SARS-CoV-2. See “HiFiViral SARS-CoV-2 Analysis” on page 43.
`ipdSummary`	Detects DNA base-modifications from kinetic signatures. See “ipdSummary” on page 46.
`isoseq3`	Characterizes full-length transcripts and generates full-length transcript isoforms, eliminating the need for computational reconstruction. See “isoseq3” on page 50.
`juliet`	A general-purpose minor variant caller that identifies and phases minor single nucleotide substitution variants in complex populations. See “juliet” on page 55.
`laa`	Finds phased consensus sequences from a pooled set of amplicons sequenced with Pacific Biosciences’ SMRT technology. See “laa” on page 62.
`Microbial Assembly`	Generates *de novo* assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb, using HiFi Reads. See “Microbial Assembly” on page 68.
`motifMaker`	Identifies motifs associated with DNA modifications in prokaryotic genomes. See “motifMaker” on page 72.
`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 74.
`pbindex`	Creates an index file that enables random access to PacBio-specific data in BAM files. See “pbindex” on page 78.
`pbmarkdup`	Marks or removes duplicates reads from CCS Reads. See “pbmarkdup” on page 78.
`pbmm2`	Aligns PacBio reads to reference sequences; a SMRT wrapper for `minimap2`. See “pbmm2” on page 80.
`pbservice`	Performs a variety of useful tasks within SMRT Link. See “pbservice” on page 87.
`pbsv`	Structural variant caller for PacBio reads. See “pbsv” on page 91.
`pbvalidate`	Validates that files produced by PacBio software are compliant with Pacific Biosciences’ own internal specifications. See “pbvalidate” on page 95.“runqc-reports” on page 97
`runqc-reports`	Generates Run QC reports. See .
`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 98.

bam2fasta/ bam2fastq

The `bam2fasta` and `bam2fastq` tools convert PacBio BAM or Data Set 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.

```
bam2fasta [options] <input>
bam2fastq [options] <input>
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.
`-o,--output`	Specifies the prefix of the output file names. − implies streaming. **Note**: Streaming is **not** supported with compression or with the `split_barcodes` option.
`-c`	Specifies the Gzip compression level. Values are `[1,2,3,4,5,6,7,8,9]`. (Default = `1`)
`-u`	Specifies that the output FASTA/FASTQ files are **not** compressed. `.gz` is not added to the output file names, and `-c` settings are ignored.
`--split-barcodes`	Splits the output into multiple FASTA /FASTQ files, by barcode pairs. **Note**: The `bam2fasta/bam2fastq` tools inspect the `bc` tag in the BAM file to determine the 0-based barcode indices from their respective positions in the barcode FASTA file.
`-p,--seqid-prefix`	Specifies the prefix for the sequence IDs used in the FAST/FASTQ file headers.

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 list of hole numbers to include or exclude, 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: **list** 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 listed 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][--include INCLUDE LIST] [--exclude EXCLUDE LIST]
                  [--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`.)
`--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`)
`--include INCLUDE LIST`	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`)
`--exclude EXCLUDE LIST`	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`)

Options	Description
`--barcodes`	Specifies that the include/exclude list contains barcode indices instead of ZMW numbers. (Default = `False`)

Examples

Pulling out two ZMWs from a BAM file:

```
bamsieve --include 111111,222222 full.subreads.bam sample.subreads.bam
```

Pulling out two ZMWs from a Data Set file:

```
bamsieve --include 111111,222222 full.subreadset.xml sample.subreadset.xml
```

Using a text list:

```
bamsieve --include zmws.txt full.subreads.bam sample.subreads.bam
```

Using another BAM or Data Set as a list:

```
bamsieve --include mapped.alignmentset.xml full.subreads.bam mappable.subreads.bam
```

Generating a list of ZMWs from a Data Set:

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

Anonymizing a Data Set:

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

Removing a read:

```
bamsieve --exclude 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 --include 4,7 full.subreads.bam two_barcodes.subreads.bam
```

Generating a tiny BAM file that contains only mappable reads:

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

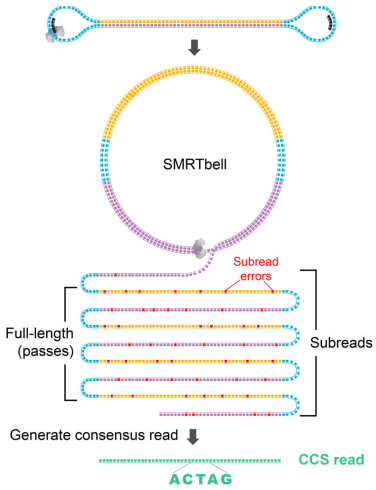
Splitting a Data Set into two halves:

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

Extracting Unmapped Reads:

```
bamsieve --exclude mapped.alignmentset.xml movie.subreadset.xml unmapped.subreadset.xml
```

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



CCS Analysis Workflow

1. **Initial Filtering**
   - Filter ZMWs: Remove ZMWs with signal-to-noise ratio (SNR) below `--min-snr`.
   - Filter subreads: Remove subreads with lengths <50% or >200% of the median subread length. Stop if the number of full-length subreads is fewer than `--min-passes`.
2. **Generate Draft**
   - The polish stage iteratively improves upon a candidate template sequence. Because polishing is very compute-intensive, it is desirable to start with a template that is as close as possible to the true sequence of the molecule to reduce the number of iterations until convergence. The `ccs` software does **not** pick a full-length subread as the initial template to be polished, but instead generates an approximate draft consensus sequence using our improved

implementation of the `Sparc` graph consensus algorithm. This algorithm depends on a subread-to-backbone alignment that is generated by the `pancake` mapper developed by PacBio, using `edlib` as the core aligner. Typically, subreads have accuracy of around 90% and the draft consensus has a higher accuracy, but is still below 99%.

- Stop if the draft length is shorter than `--min-length` and longer than `--max-length`.

3. **Alignment:**
   - Align subreads to the draft consensus using `pancake` with KSW2 for downstream windowing and filtering.
4. **Windowing**
   - Divide the subread-to-draft alignment into overlapping windows with a target size of 22 bp with ±2 bp overlap. Avoid breaking windows at simple repeats (homopolymers to 4-mer repeats) to reduce edge cases at window borders. Windowing reduces the algorithm run time from quadratic to linear in the insert size.
5. **Single-Strand Artifacts**
   - Identify heteroduplexes, where one strand of the SMRTbell differs significantly from the reverse complement of the other strand. Subread orientation is inferred from the alignment. Small heteroduplexes, such as single base A paired with a matching G, are retained and the ambiguity is reflected in base quality. Molecules with a single difference longer than 20 bp between the strands are removed and recorded as heteroduplexes in the `<outputPrefix>.ccs_report.txt` file.
6. **Trim Large Insertions**
   - Insertions in the subreads relative to the draft that are longer than `--max-insertion-size`, default 30 bp, are trimmed since they typically represented spurious sequencing activity.
7. **Filter Candidates**
   - For each window, a heuristic is used to find those positions that likely need polishing. In addition, homopolymers are always polished. Skipping unambiguous positions makes the polishing at least twice as fast.
8. **Polishing**
   - The core polishing uses the `arrow` algorithm, a left-right Hidden Markov-Model (HMM) that models the enzymatic and photophysical events in SMRT Sequencing. Emission and transition parameters are estimated by a dinucleotide template context. Transition parameters form a homogeneous Markov chain. The transition parameters do **not** depend on the position within the template, only the pulse width of a base call, the dinucleotide context of the template, and the SNR of the ZMW. `Arrow` computes the log-likelihood that the subread originates from the template, marginalizing over all possible alignments of the subread to the template. For every position in the template that is a candidate for polishing, `arrow` tests if the log-likelihood is improved by substituting

one of the other three nucleotides, inserting one of the four nucleotides after the position, or deleting the position itself. Once `arrow` does not find any further beneficial mutations to the template in an iteration, it stops.

9. **QV Calculation**
   - The log-likelihood ratio between the most likely template sequence and all of its mutated counterparts is used to calculate a quality for each base in the final consensus. The average of the per-base qualities is the read accuracy, `rq`.
10. **Final Steps**
   - The per-window consensus template sequences and base qualities are concatenated and overhangs (overlaps between adjacent windows) are trimmed. If the predicted read accuracy is at least `--min-rq`, then the consensus read is written to the output.

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 productive ZMW. BAM is a general file format for storing sequence data, which is described fully by the SAM/BAM working group. The CCS Analysis 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.

```
m64009_201008_223950/1/ccs    4    *    0    255    *    *    0    0    ATCGCCTACC
~|~t~R~~r~    RG:Z:a773c1f2    fi:B:C,26,60,21,41,33,26,63,45,73,33    fn:i:6
fp:B:C,11,18,21,35,8,18,31,8,23,11    np:i:12
ri:B:C,17,37,24,4,70,21,12,44,21,32    rn:i:6    rp:B:C,16,56,17,9,23,19,10,10,23,12
  rq:f:0.999651    sn:B:f,10.999,16.2603,3.964,7.17746    we:i:9816064    ws:i:20
zm:i:1
```

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`.

Field	Description
TLEN	Required by the format but meaningless in this context. Always set to 0.
Consensus Sequence	The consensus sequence generated.
Quality Values	The per-base parametric quality metric. For details see “Interpreting QUAL Values” on page 11.
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.
zm Tag	The ZMW hole number.

Usage

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

Example

```
ccs --all 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)
--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)

Options	Description
`--min-rq`	Specifies the minimum predicted accuracy of a read. CCS Analysis 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 Analysis uses 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.
`--model-path`	Specifies the path to a model file or directory containing model files.
`--model-spec`	Specifies the name of the chemistry or model to use, overriding the default selection.
`--all`	Generates **one** representative sequence per ZMW, irrespective of quality and passes. `--min-passes 0 --min-rq 0 --max-length 0` are set implicitly and **cannot** be changed; `--all` also deactivates the maximum draft length filter. Filtering has to be performed downstream. The `ccs --all` option changes the workflow as follows: 1. There is special behavior for low-pass ZMWs. If a ZMW has fewer than 2 full-length subreads, use the subread of median length as representative consensus, optionally with its kinetic information as forward orientation using `--all-kinetics`, and do not polish. 2. Only polish ZMWs with at least two full-length subreads mapping back to the draft. Otherwise, set predicted accuracy `rq` tag to `-1` to indicate that the predicted accuracy was not calculated, and populate per-base QVs with + (QV 10) the approximate raw accuracy. Kinetic information is **not** available for unpolished drafts. 3. Instead of using an unpolished draft without kinetic information as a representative consensus sequence, if `--subread-fallback` is used, fall back to a representative subread with kinetic information. How is `--all` different from explicitly setting `--min-passes 0 --min-rq 0`? • Setting `--min-passes 0 --min-rq 0` is a brute-force combination that polishes **every** ZMW, even those that only have one partial subread, with polishing making no difference. In contrast, `--all` is a bit smarter and only polishes ZMWs with at least one full-length subread and one additional partial subread.

Options	Description
`--hifi-kinetics`	Generates averaged kinetic information for polished reads, independently for both strands of the insert. Forward is defined with respect to the orientation represented in SEQ and is considered to be the native orientation. As with other PacBio-specific tags, aligners will **not** re-orient these fields. Base modifications can be inferred from per-base pulse width (PW) and inter-pulse duration (IPD) kinetics. Minor cases exist where a certain orientation may get filtered out entirely from a ZMW, preventing valid values from being passed for that record. In these cases, empty lists are passed for the respective record/orientation, and number of passes are set to zero. To facilitate the use of HiFi Reads with base modifications workflows, we added an executable in `pbbam` called `ccs-kinetics-bystrandify` which creates a pseudo `--by-strand` BAM with corresponding `pw` and `ip` tags that imitates a normal, unaligned subreads BAM.
`--all-kinetics`	Adds kinetic information for **all** ZMWs, except for unpolished draft consensus.
`--subread-fallback`	When combined with `--all`, uses a subread instead of a draft as representative consensus.
`--suppress-reports`	Suppresses the generation of default reports and metric files.

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 an `*` 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 Analysis Yield Report

The CCS Analysis Yield 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	None	The number of input ZMWs.
ZMWs pass filters	All custom processing settings	The number of CCS Reads successfully produced on the first attempt, using the fast windowed approach.
ZMWs fail filters	All custom processing settings	The number of ZMWs reads that failed to produce a CCS Read.
ZMWs shortcut filters	`-all`	The number of ZMWs having fewer than 2 full-length subreads.
ZMWs with tandem repeats	`--min-tandem-repeat-length`	The number of ZMWs with repeats larger than the specified threshold.

The second part explains in detail the exclusive ZMW count for those ZMWs that were filtered:

ZMW Results	Parameters Affecting Results	Description
No usable subreads	None	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.
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.

ZMW Results	Parameters Affecting Results	Description
CCS Analysis did not converge	None	The consensus sequence did not converge after the maximum number of allowed rounds of polishing.
CCS Read 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.

How do I read the ccs_report.txt file?

By default, each CCS Analysis generates a `ccs_report.txt` file. This file summarizes how many ZMWs generated HiFi Reads and how many ZMWs failed CCS Reads generation because of the listed causes. For those failing, each ZMW contributes to exactly one reason of failure; percentages are with respect to number of failed ZMWs.

Does CCS Analysis dislike low-complexity regions?

Low-complexity comes in many shapes and forms. A particular challenge for CCS Analysis are highly-enriched tandem repeats, such as hundreds of copies of AGGGGT. Prior to `ccs` v5.0, inserts with many copies of a small repeat likely did not generate a consensus sequence. Since `ccs` v5.0, every ZMW is tested if it contains a tandem repeat of length `--min-tandem-repeat-length 1000`. For this, we use symmetric DUST, specifically this `sdust` implementation, but slightly modified. If a ZMW is flagged as a tandem repeat, internally `--disable-heuristics` is activated for only this ZMW, and various filters that are known to exclude low-complexity sequences are disabled. This recovers most of the low-complexity consensus sequences, without impacting run time performance.

Can I produce one consensus sequence for each strand of a molecule?

Yes, use `--by-strand`. Make sure that you have sufficient coverage, as `--min-passes` are per strand in this case. For each strand, CCS Analysis generates one consensus read that has to pass all filters. Read name suffix indicates strand. Example:

```
m64011_190714_120746/14/ccs/rev
m64011_190714_120746/35/ccs/fwd
```

How does `--by-strand` work? For each ZMW:

- Determine orientation of reads with respect to the one closest to the median length.
- Sort reads into two buckets, forward and reverse strands.
- Treat each strand as an individual entity as we do with ZMWs:

- Apply all filters per strand individually.
- Create a draft for each strand.
- Polish each strand.
- Write out each polished strand consensus.

BAM Tags Generated

Tag	Type	Description
ec	f	Effective coverage
fi	B,C	Forward IPD (Codec V1)
fn	i	Forward number of complete passes (zero or more)
fp	B,C	Forward PulseWidth (Codec V1)
np	i	Number of full-length subreads
ri	B,C	Reverse IPD (Codec V1)
rn	i	Reverse number of complete passes (zero or more)
rp	B,C	Reverse PulseWidth (Codec V1)
rq	f	Predicted average read accuracy
sn	B,F	Signal-to-noise ratios for each nucleotide
zm	i	ZMW hole number
RG	z	Read group

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)

Options	Description
-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: alignedlength, as, astart, bc, bcf, bcq, bcr, bq, cx, length, mapqv, movie, n_subreads, pos, qend, qid, 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. The filter string should be enclosed in **single quotes**.

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

Examples

Filter on read quality > 0.99 (Q20):

```
% dataset filter in.consensusreadset.xml hifi.consensusreadset.xml 'rq >= 0.99'
```

Filter on read quality and length:

```
% dataset filter in.consensusreadset.xml filtered.consensusreadset.xml 'rq >= 0.99 AND
length >= 10000'
```

Filter for very long and very short reads:

```
% dataset filter in.consensusreadset.xml filtered.consensusreadset.xml 'length >=
40000; length <= 400'
```

Filter for specific high-quality barcodes:

```
% dataset filter mixed.consensusreadset.xml samples1-3.consensusreadset.xml 'bc =
[0,1,2] AND bq >= 26'
```

`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 sub-datasets. (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.

Examples - 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
```

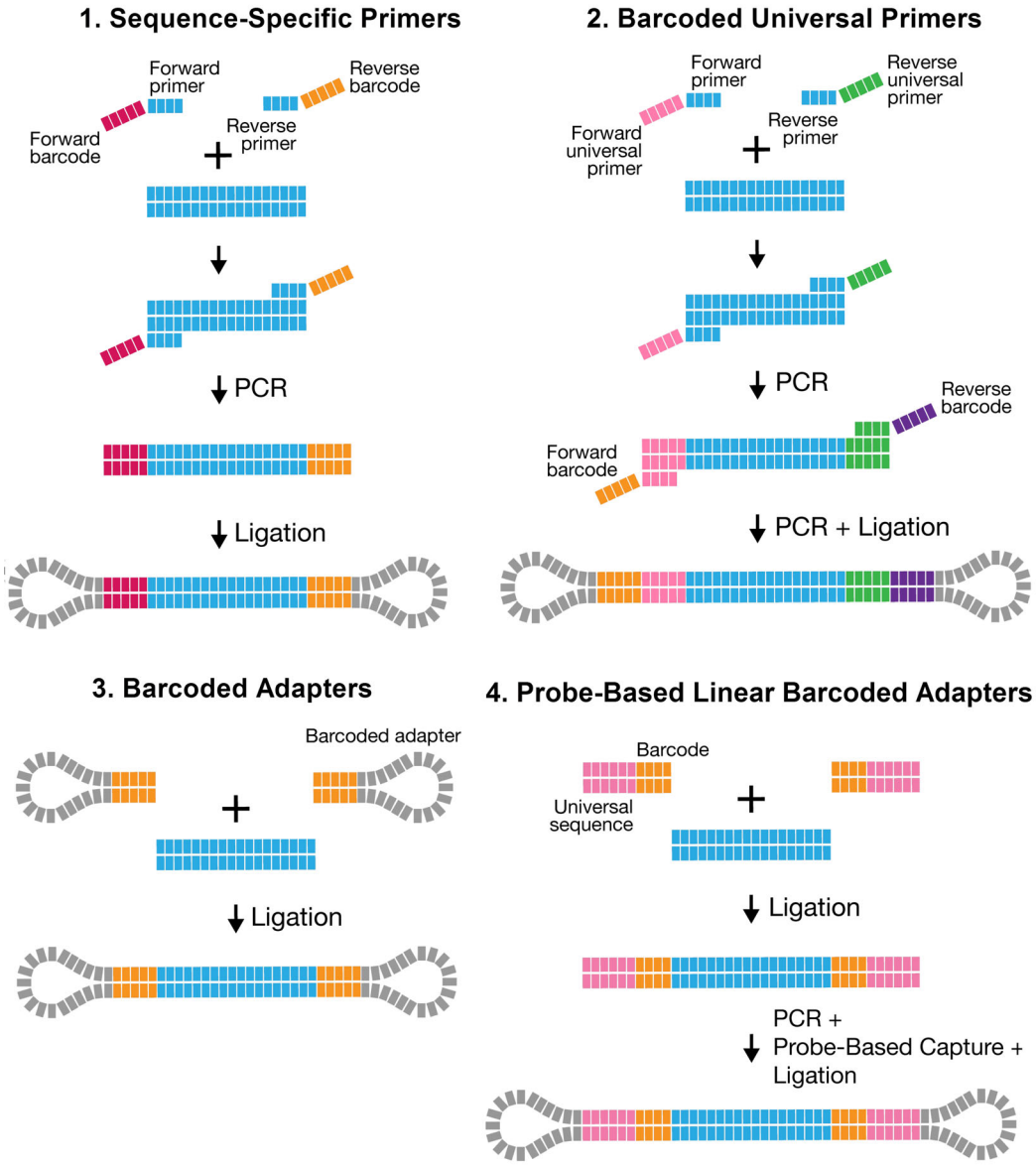
The chunking works by duplicating the original merged Data Set (no BAM duplication) and adding filters to each duplicate such that only reads belonging to the appropriate contigs are emitted. The contigs are distributed among the output files in such a way that the total number of records per chunk is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

Demultiplex Barcodes

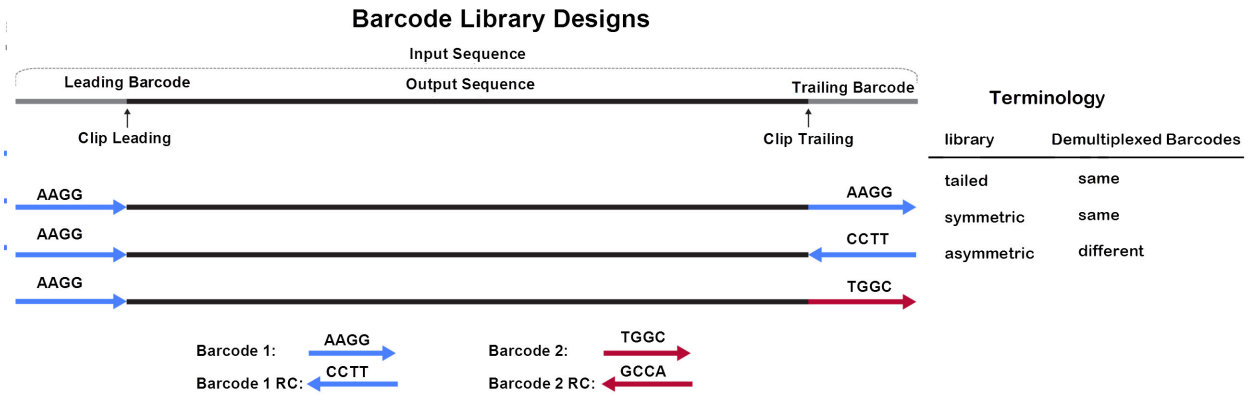
The **Demultiplex Barcodes** application identifies barcode sequences in PacBio single-molecule sequencing data. It **replaced** `pbbarcode` and `bam2bam` for demultiplexing, starting with SMRT® Analysis v5.1.0.

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 Reads 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.

Parameter Presets

Recommended parameter combinations are available using `--preset` for HiFi input:

- HIFI-SYMMETRIC
  `--ccs --min-score 80 --min-end-score 50 --min-ref-span 0.75 --same`
- HIFI-ASYMMETRIC
  `--ccs --min-score 80 --min-end-score 50 --min-ref-span 0.75 --different --min-scoring-regions 2`
- NONE (Default)

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 Reads:

```
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 Reads: --same --ccs
```

Asymmetric options:

```
Raw: --different
CCS Reads: --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`.
`--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`)

Options	Description
`--peek-guess`	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.
`--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 30 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 Sequel Systems.
- Unaligned CCS Reads, generated by CCS Analysis.

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.count`

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 application, **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 Reads BAM file for an Iso-Seq Data Set before running `lima`. The recommended parameters for running CCS Analysis for Iso-Seq are `min-pass=1`, `min accuracy=0.9`, 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 Iso-Seq v2 primer set (included with the SMRT Link installation).

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

Note: The Clontech kit is **unsupported,** and these primers will **not** be included in future SMRT Link releases. We recommend using the NEBNext® Single Cell/Low Input cDNA Synthesis & Amplification Module.

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

```
>NEB_5p
GCAATGAAGTCGCAGGGTTGGG
>dT_BC1001_3p
AAGCAGTGGTATCAACGCAGAGTACCACATATCAGAGTGCG
>dT_BC1002_3p
AAGCAGTGGTATCAACGCAGAGTACACACACAGACTGTGAG
>dT_BC1003_3p
AAGCAGTGGTATCAACGCAGAGTACACACATCTCGTGAGAG
>dT_BC1004_3p
AAGCAGTGGTATCAACGCAGAGTACCACGCACACACGCGCG
```

Note: NEB_5p is **not** an NEB primer sequence; it is PacBio's Iso-Seq Express cDNA PCR primer sequence listed in the protocol.

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.

BAM Tags

In SMRT Link v8.0 and earlier, no `LB` and `SM` tags were written the BAM file. In SMRT Link v10.2, `LB` and `SM` tags are set by the user in Run Design. The `SM` tag can also be set in Demultiplex Barcodes in SMRT® Analysis.

Non-demultiplex case:

- `LB`: Well Sample Name.
- `SM`: Bio Sample Name.

Multiplexed case, BAM pre-demultiplexing:

- `LB`: Well Sample Name.
- `SM`: Tag removed.

Multiplexed case, BAMs post-demultiplexing:

- `LB`: Well Sample Name for all child barcode BAMs.
- `SM`: Each individual Bio Sample Name for the specific barcode.
- `BC`: Barcode sequence or hyphenated barcode sequences of the pair.
- `DS`: Appends barcode information used in demultiplexing: BarcodeFile, BarcodeHash, BarcodeCount, BarcodeMode, BarcodeQuality.
- Example read group header after demultiplexing:

```
@RG
ID:66d5a6af/3--3
PL:PACBIO
DS:READTYPE=SUBREAD;
   Ipd:CodecV1=ip;
   PulseWidth:CodecV1=pw;
   BINDINGKIT=101-500-400;
   SEQUENCINGKIT=101-427-800;
   BASECALLERVERSION=5.0.0;
   FRAMERATEHZ=100.000000;
   BarcodeFile=Sequel_16_barcodes_v3.barcodeset.xml;
   BarcodeHash=f2b1fa0b43eb6ccbb30749883bb550e3;
   BarcodeCount=16;
   BarcodeMode=Symmetric;
   BarcodeQuality=Score
PU:m54010_200212_162236
SM:MySampleName
PM:SEQUEL
BC:ACAGTCGAGCGCTGCGT
```

export-datasets

The `export-datasets` tool takes one or more PacBio Data Set XML files and packages all contents (including index files and supplemental Data Sets) into a single ZIP archive. Data Set resources, such as BAM files, are reorganized and renamed to flatten the directory structure, avoid redundant file writes, and convert all resource paths from absolute paths to relative paths. Where multiple Data Sets are provided, the contents of each is nested in a directory named after the `UniqueId` attribute in the XML.

The resulting archive is primarily intended to be directly imported into SMRT Link using the Data Management interface, but it may also be unpacked manually and used on the command line.

Usage

```
export-datasets [options] <dataset>...
```

Options	Description
`-o, --output`	Name of output ZIP file. (Default = `datasets_<timestamp>.zip`)
`--keep-parent-ref`	Keeps the reference to the parent Data Set when archiving a demultiplexed child Data Set.
`--no-scraps`	Excludes the `scraps.bam` file if present in the XML file.
`-h, --help`	Displays help information and exits.
`--log-file`	Writes the log to a file. (Default = `stderr`)
`--log-level`	Specifies the log level; values are `[ERROR, DEBUG, INFO, WARN]`. (Default = `WARN`)
`--logback`	Override all logger configuration using a specified `logback.xml` file.
`--log2stdout`	If `True`, log output is displayed to the console. (Default = `False`)
`--debug`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet`	Alias for setting the log level to `ERROR`. (Default = `False`)
`--verbose`	Alias for setting the log level to `INFO`. (Default = `False`)

Input Files

- One or more PacBio Dataset XML files.

Output File

- One output ZIP file.

Examples

```
export-datasets m64001_200704_012345.subreadset.xml

export-datasets sample1.consensusreadset.xml sample2.consensusreadset.xml
\sample3.consensusreadset.xml -o barcoded_ccs.zip

export-datasets /opt/smrtlink/jobs/0000/0000001/0000001234/outputs/
mapped.alignmentset.xml
```

export-job

The `export-job` tool packages a SMRT Link Analysis job for export to another system, usually for reimportation into another SMRT Link instance. All internal paths in job output files are converted from absolute to relative paths, and many of the internal details of the Cromwell workflows are omitted. The export is **not** a complete record of the job, but rather a collection of job output files and metadata.

Note that `export-job` **will** include any external Data Sets referenced in output Data Sets inside the job, for example ReferenceSets associated with mapped Data Sets, or BarcodeSets associated with demultiplexed Data Sets. However, these Data Sets will **not** be imported along with the job. The exported job does **not** include the input reads used to run the job; these may be exported separately using the `export-datasets` tool.

IMPORTANT: Only SMRT Link v10.0 or later generates the necessary metadata files for `export-job` to save a full record of job execution. Jobs created with older versions of SMRT Link will still be archived, but the metadata will be empty and/or incorrect.

Usage

```
export-job [options] <job_dir>
```

Options	Description
`<job_dir>`	Path to a SMRT Link job directory.
`-o, --output`	Name of output ZIP file. (Default = `job_<timestamp>.zip`)
`-h, --help`	Displays help information and exits.
`--log-file`	Writes the log to a file. (Default = `stderr`)
`--log-level`	Specifies the log level; values are `[ERROR, DEBUG, INFO, WARN]`. (Default = `WARN`)
`--logback`	Override all logger configuration using a specified `logback.xml` file.
`--log2stdout`	If `True`, log output is displayed to the console. (Default = `False`)
`--debug`	Alias for setting the log level to `DEBUG`. (Default = `False`)
`--quiet`	Alias for setting the log level to `ERROR`. (Default = `False`)
`--verbose`	Alias for setting the log level to `INFO`. (Default = `False`)

Input

- A path to a job directory.

Output File

- One output ZIP file.

Examples

```
export-job /path/to/smrtlink/jobs-root/0000/0000000/0000000860 -o job860.zip
```

To reimport on another system:

```
pbservice import-job job860.zip
```

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. If specifying an input FASTA file, a FASTA index file (`.fai`) with the same name and path is **required**. If the `.fai` file is not supplied, `gcpp` exits and displays an error message.

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`, `arrow` and `poa`. (Default = `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.

Genome Assembly

The Genome Assembly application generates *de novo* assemblies using HiFi Reads. The application is fast, produces contiguous assemblies, and is suitable for genomes of any size.

The Genome Assembly application is powered by the IPA HiFi genome assembler and includes the following features:

- Separates haplotypes during assembly using a novel phasing stage (Nighthawk).
- Polishes the contigs with phased reads using `Racon`.
- Improves haplotype separation using the `purge_dups` tool.

Workflow of the Genome Assembly Application

Analysis steps are highly optimized to produce assemblies of large genomes efficiently.

The workflow consists of seven stages:

1. Sequence database construction.
2. Fast overlap computation using the `Pancake` tool.
3. A dedicated phasing stage using the `Nighthawk` tool.
4. Filtering chimeras and residual repeats.
5. Layout based on the string graph.
6. Polishing using the `Racon` tool.
7. Purging haplotype duplicates from the primary assembly using the third-party tool `purge_dups`.

The workflow accepts HiFi XML Data Sets as input.

IPA HiFi Genome Assembler

- Scales well on a cluster.
- The workflow has an embedded downsampling feature:
  - If the genome size and the desired coverage are specified, the initial stage (SeqDB construction) downsamples the input Data Set to the desired coverage.
  - Otherwise, the full coverage is used.

Usage

The Genome Assembly application is run using the `pbcromwell run` command, with the `pb_assembly_hifi` parameter to specify the application. See "pbcromwell" on page 74 for details.

To view information on the available Genome Assembly options, enter:

```
pbcromwell show-workflow-details pb_assembly_hifi
```

The **minimum** command needed to run the workflow requires the input and the number of threads. The following example uses 16 threads:

```
pbcromwell run pb_assembly_hifi -e <input.xml> --nproc 16
```

The following example performs an assembly using an input XML Data Set, and uses all default settings, including 1 CPU:

```
pbcromwell run pb_assembly_hifi -e <input.consensusreadset.xml>
```

Note: The default options for this workflow are equivalent to the following command:

```
pbcromwell run pb_assembly_hifi \
-e <input.consensusreadset.xml> \
--task-option reads=None \
--task-option ipa2_genome_size=0 \
--task-option ipa2_downsampled_coverage=0 \
--task-option ipa2_advanced_options="" \
--task-option ipa2_run_polishing=True \
--task-option ipa2_run_phasing=True \
--task-option ipa2_run_purge_dups=True \
--task-option ipa2_ctg_prefix="ctg." \
--task-option ipa2_reads_db_prefix="reads" \
--task-option ipa2_cleanup_intermediate_files=True \
--task-option dataset_filters="" \
--task-option filter_min_qv=20 \
--nproc 8
```

The default options for this workflow should work well for any genome types.

If the assembly is run on a single local node with high CPU count, such as 64 cores, we recommend that the job submission for `pbcromwell` is configured so that it uses 4 concurrent jobs and 16 threads per job.

We found this to be more efficient than using 64 threads and 1 concurrent job, as many steps are very data I/O-dependent.

You can apply a similar principle for compute environments with more or fewer cores. For example, for a machine with 80 cores, one can use 20 threads and 4 concurrent jobs.

Genome Assembly Parameters Input Files

Option	Default Value	Description
`-e, --eid_ccs`	NONE	Optional parameter, required if `--task-option reads <input>` is **not** specified. This is a SMRT Link-specific input parameter and supports **only** PacBio `Consensusreadset` XML files as input.
`--task-option reads`	NONE	Optional parameter, required if `-e <input>` is **not** specified. Supports multiple input formats: FASTA, FASTQ, BAM, XML, FOFN and gzipped versions of FASTA/FASTQ.
`--task-option ipa2_genome_size`	0	The approximate number of base pairs expected in the genome. This is used only for downsampling; if the value is ≤ `0`, downsampling is disabled. **Note**: It is better to slightly overestimate rather than underestimate the genome length to ensure good coverage across the genome.
`--task-option ipa2_downsampled_coverage`	0	The input Data Set can be downsampled to a desired coverage, provided that both the `ipa2_downsampled_coverage` and `ipa2_genome_size` options are specified and >`0`. Downsampling applies to the **entire** assembly process, including polishing. This parameter selects reads randomly, using a fixed random seed for reproducibility.
`--task-option ipa2_advanced_options`	NONE	A semicolon-separated list of KEY=VALUE pairs. New line characters are not accepted. (These are described later in this document.)
`--task-option ipa2_run_polishing`	TRUE	Enables or disables the polishing stage of the workflow. Polishing can be disabled to perform fast draft assemblies.
`--task-option ipa2_run_phasing`	TRUE	Enables or disables the phasing stage of the workflow. Phasing can be disabled to assemble haploid genomes, or to perform fast draft assemblies.
`--task-option ipa2_run_purge_dups`	TRUE	Enables or disables identification of “duplicate” alternate haplotype contigs which may be assembled in the primary contig file, and moves them to the associate contig (haplotig) file.
`--task-option ipa2_ctg_prefix`	`.ctg`	The prefix used to label the output generated contigs.
`--task-option ipa2_reads_db_prefix`	`reads`	The prefix of the sequence and seed databases which will be used internally for assembly.
`--task-option ipa2_cleanup_intermediate_files`	TRUE	Removes intermediate files from the run directory to save space.
`--task-option dataset_filters`	NONE	**(General pbcromwell option)** A semicolon-separated (**not** comma-separated) list of other filters to add to the Data Set.
`--task-option filter_min_qv`	20	**(General pbcromwell option)** Phred-scale integer QV cutoff for filtering HiFi Reads. The default for all applications (**except** Iso-Seq analysis) is `20` (QV 20), or 99% predicted accuracy.

Option	Default Value	Description
`--config`	NONE	**(General pbcromwell option)** Java configuration file for running Cromwell.
`--nproc`	1	**(General pbcromwell option)** Number of processors per task.

- `*.bam` file containing PacBio data.
- `*.fasta` or `*.fastq` file containing PacBio data.
- `*.xml` file containing PacBio data.
- `*.fofn` files with file names of files containing PacBio data.

Output Files

- `final_purged_primary.fasta` file containing assembled primary contigs.
- `final_purged_haplotigs.fasta` file containing assembled haplotigs.

Advanced Parameters

Advanced parameters should be rarely modified. For the special cases when that is required, advanced parameters are documented below.

Advanced parameters specified on the command line:

- Are in the form of `key = value` pairs.
- Each pair is separated by a semicolon (`;`) character.
- The full set of advanced parameters is surrounded by **one** set of double quotes.
- The specified value of a parameter **overwrites** the default options for that key **– all** desired options of that parameter must be explicitly listed, not just the ones which should change from the default.
- Setting an empty value **clears** the parameter; it does **not** reset the value back to default.

Example:

```
--task-option ipa2_advanced_options="config_seeddb_opt=-k 30;config_block_size=2048"
```

Complete List of Available Advanced Parameters and Default Values:

Advanced Parameters	Default Value	Description
`config_genome_size`	0	The approximate number of base pairs expected in the genome, used to determine the coverage cutoff. This is only used for downsampling; `0` turns downsampling off. **Note**: It is better to slightly overestimate rather than underestimate the genome length to ensure good coverage across the genome.

Advanced Parameters	Default Value	Description
config_coverage	0	The input Data Set can be downsampled to a desired coverage, provided that both the Downsampled coverage and Genome Length parameters are specified and above 0. Downsampling applies to the **entire** assembly process, including polishing. This feature selects reads randomly, using a fixed random seed for reproducibility.
config_polish_run	1	Enables or disables the polishing stage of the workflow. Polishing can be disabled to perform fast draft assemblies. 0 disables this feature; 1 enables it.
config_phase_run	1	Enables or disables the phasing stage of the workflow. Phasing can be disabled to assemble haploid genomes, or to perform fast draft assemblies. 0 disables this feature; 1 enables it.
config_purge_dups_run	1	Enables or disables the purge_dups stage of the workflow. 0 disables this feature; 1 enables it.
config_autocomp_max_cov	1	If enabled, the maximum allowed overlap coverage at either the 5' or the 3' end of every read is automatically determined based on the statistics computed from the overlap piles. This value is appended to the config_ovl_filter_opt value internally, and supersedes the manually specified --max-cov and --max-diff values of that parameter. These options are used to determine potential repeats and filter out those reads before the string graph is constructed. 0 disables this feature; 1 enables it.
config_block_size	4096	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.
config_existing_db_prefix	NONE	Allows injection of an existing SeqDB, so that one doesn't have to be built from scratch. The provided existing DB is symbolically linked and used for assembly. (This option is intended for debugging purposes.)

Advanced Parameters	Default Value	Description
config_ovl_filter_opt	--max-diff 80 --max-cov 100 --min-cov 2 --bestn 10 --min-len 4000 --gapFilt --minDepth 4 --idt-stage2 98	Overlap filter options. --gapFilt - Enables the chimera filter, which analyzes each 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 tangles 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, we advise that this value be also increased. Alternatively, using the autocompute_max_cov option can automatically estimate the value of this parameter, which can improve contiguity (for example, in cases when the input genome size or the seed coverage were overestimated). --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. If the autocompute_max_cov option is used, then the same computed value is supplied to this parameter as well. --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. --idt-stage2 - Filter overlaps with identity below 98%. --high-copy-sample-rate - Controls the downsampling of reads from high copy elements to the expected coverage determined by maxCov*rate, where rate is the value of this parameter. If rate is 0, then these high coverage reads are discarded.
config_ovl_min_idt	98	The final overlap identity threshold. Applied during the final filtering stage, right before the overlaps are passed to the layout stage.
config_ovl_min_len	1000	The minimum length of either A-read or a B-read to keep the overlap. Applied during the final filtering stage, right before the overlaps are passed to the layout stage.
config_ovl_opt	--one-hit-per-target --min-idt 96	Overlapping options for the pancake overlapping tool. The options set by this parameter here are passed directly to pancake. For details on pancake options, use pancake -h. The defaults used here are: --one-hit-per-target which keeps only the best hit in case there are multiple possible overlaps between a pair of reads (tandem repeats); and --min-idt 96 which will filter out any overlap with identity lower than 96%.
config_phasing_opt	NONE	Options for the phasing tool nighthawk. The options set by this parameter are passed directly to nighthawk. For details on nighthawk options, use nighthawk -h.

Advanced Parameters	Default Value	Description
config_phasing_split_opt	--split-type noverlaps --limit 3000000	Options that control the chunking of the phasing jobs, and through that regulate the time and memory consumption of each individual chunk. The defaults are: --split-type noverlaps which splits the chunks by the number of overlaps; and --limit 3000000 which allow at most approximately 3 million overlaps per chunk. Empirically, the current defaults keep the maximum memory consumption (RSS) of the phasing jobs under 4 GB per chunk.
config_seeddb_opt	-k 28 -w 120 --space 1	Options to control the seed computation. These options are passed directly to the pancake seeddb command. Defaults: -k 28 is the k-mer size of 28 bp; -w 120 is the minimizer window size of 120 bp; and --space 1 specifies the spacing for spaced seed construction, with 1 gap in between every two bases of the seed. For more details on these and other options, use pancake seeddb -h.
config_seqdb_opt	--compression 1	Options to control the construction of the sequence database. These options are passed directly to the pancake seqdb command. Current default is --compression 1 which turns on the 2-bit encoding compression of the sequences. For more details on these and other options, use pancake seqdb -h.
config_use_hpc	0	This parameter enables (1) or disables (0) an experimental Homopolymer Compression feature. If this feature is enabled, the overlaps are computed from homopolymer-compressed sequences. The layout stage is somewhat slower because the sequences have to be aligned to determine the correct homopolymer-expanded coordinates.
config_use_seq_ids	1	This feature is mostly useful for debugging purposes. If 0 is specified, then the overlaps contain original sequence names instead of their numerical IDs. The default of 1 uses the numerical IDs to represent reads, which uses memory much more efficiently.
config_purge_map_opt	--min-map-len 1000 --min-idt 98.0 --bestn 5	This option is used to control the mapping of the reads to contigs for the purge_dups tool. The mapper used is pancake, and the options set by this parameter are used directly by pancake. For details on pancake options, use pancake -h. Option --min-map-len 1000 removes any alignment which did not span more than 1000 bp during the mapping process; --min-idt 98.0 removes any alignment with identity below 98.0%, and --bestn 5 keeps at most 5 top scoring alignments for each query read (one primary alignment and at most 4 secondary alignments).

Advanced Parameters	Default Value	Description
config_purge_dups_calcuts	NONE	The third-party tool purge_dups can accept user-defined cutoffs for purging. On some genomes, the automated computation of the cutoffs in purge_dups can result in suboptimal values, and in this case a user can specify them manually. This option is passed directly to the purge_dups_calcuts tool. For details on the possible values that can be passed to this tool, use ipa_purge_dups_calcuts without parameters. Relevant parameters include: -l INT Lower bound for read depth. -m INT Transition between haploid and diploid. -u INT Upper bound for read depth.
config_m4filt_high_copy_sample_rate	1.0	This option is passed to the --high-copy-sample-rate parameter of the overlap filter, which controls the downsampling of reads from high copy elements to the expected coverage determined by maxCov*rate, where rate is the value of this parameter. If 0, then these high coverage reads are discarded. **Note**: This parameter supersedes the config_ovl_filter_opt options.
config_max_polish_block_mb	100	During the polishing stage, contigs are grouped into chunks of approximate size specified by this parameter (in megabases). Each chunk is processed separately and in parallel (depending on the system configuration).
config_layout_opt	NONE	This value is passed directly to the assembly layout stage. To get a list of valid options for this parameter, enter ipa2_ovlp_to_graph -h on the command line.

HiFiViral SARS-CoV-2 Analysis

Use this application to analyze multiplexed samples sequenced with the HiFiViral SARS-CoV-2 Kit. For **each** sample, this analysis provides:

- Consensus sequence (FASTA)
- Variant calls (VCF)
- HiFi Reads aligned to the reference (BAM)
- Plot of HiFi Read coverage depth across the SARS-CoV-2 genome.

Across **all** samples, this analysis provides:

- Job summary table including passing sample count at 90 and 95% genome coverage.
- Sample summary table including, for each sample: Count of variable sites, genome coverage, read coverage, and probability of multiple strains, and other metrics.
- Plate QC graphical summary of performance across samples in assay plate layout.
- Plot of HiFi Read depth of coverage for all samples.

Notes:

- The application accepts **HiFi Reads** (BAM format) as input. **HiFi Reads** are reads generated with CCS Analysis that have a quality value equal to or greater than Phred-scaled Q20.
- This application is for SARS-CoV-2 analysis **only** and is **not** recommended for other viral studies. The Wuhan reference genome is included with SMRT Link and used by default, but advanced users may specify other reference genomes. We have **not** tested the application with reference genomes other than the Wuhan reference genome.
- The application is intended to identify variable sites and call a single consensus sequence per sample. The output consensus sequence is produced based on the dominant variant observed. Minor variant information that passes through a default threshold may be encoded in the raw VCF, but does **not** get propagated into the consensus sequence FASTA.
- The HiFiViral SARS-CoV-2 Analysis application can be run using the **Auto Analysis** feature available in Run Design. This feature allows users to complete all necessary analysis steps immediately after sequencing **without** manual intervention. The Auto Analysis workflow includes CCS, Demultiplex Barcodes, and HiFiViral SARS-CoV-2 Analysis.

HiFiViral SARS-CoV-2 Application Workflow

1. Process the reads using the `mimux` tool to trim the probe arm sequences.
2. Align the reads to the reference genome using `pbmm2`.
3. Call and filter variants using `bcftools`, generating the raw variant calls in VCF file format. Filtering in this step removes low-quality calls (less than Q20), and normalizes indels.
4. Filter low-frequency variants using `vcfcons` and generate a consensus sequence by injecting variants into the reference genome. At each position, a variant is called **only** if **both** the base coverage **exceeds** the minimum base coverage threshold (Default = 4) **and** the fraction of reads that support this variant is **above** the minimum variant frequency threshold (Default = 0.5). See here for details.

Preparing Input Data for the HiFiViral SARS-CoV-2 Analysis Application

1. Run the **Demultiplex Barcodes** `cromwell` workflow, where the input to that application are HiFi Reads, and the primers are multiplexed barcode primers. See “Demultiplex Barcodes” on page 20 for details. If HiFi Reads have **not** been generated on the instrument, run CCS Analysis first. See “ccs” on page 6 for details.
   - Provide the proper barcode sequences: `HiFiViral_SARS-CoV-2_M13barcodes`.
   - Use the task option `lima_symmetric_barcodes=false`. (The barcode pairs are **asymmetric**.)

- Provide the correctly-formatted barcode pair-to-Bio Sample CSV file. For details, see the `--task-option sample_wells_csv` option in the table further down this page.

Input Files

- `movie.consensusreadset.xml`: Previously-demultiplexed HiFi Reads, packaged as separate BAM files wrapped in an XML Data Set. (See "Preparing Input Data for the HiFiViral SARS-CoV-2 Analysis Application" on page 44 for details.)
- `sars_cov2.referenceset.xml`: The Wuhan reference genome.
- `HiFiViral_SARS-CoV-2_Enrichment_Probes.barcodeset.xml`: Dataset XML specifying the probe sequence file in FASTA format.
- [**Optional**] `Plate QC csv`: Four-column CSV file that includes `barcode`, `biosample`, `plateID` and `wellID`.
  To specify this optional file, add the following option to `pbservice`:
  `--task-option sample_wells_csv=<path to CSV file>`

Output Files

- `pb_sars_cov2_kit.probe_counts_zip`: Zipped TSV files with probe counts, per sample.
- `pb_sars_cov2_kit.variants_csv`: CSV variant calls for **all** samples.
- `pb_sars_cov2_kit.vcf_zip`: Zipped VCF files containing the final variant calls, per sample.
- `pb_sars_cov2_kit.raw_vcf_zip`: Zipped VCF files containing the raw variant calls, per sample.
- `pb_sars_cov2_kit.fasta_zip`: Zipped final consensus sequences, by sample, in FASTA format. This is a single consensus sequence with `N`s for each sample.
- `pb_sars_cov2_kit.frag_fasta_zip`: Zipped file of consensus sequences, split on `N`s, in FASTA format, by sample.
- `pb_sars_cov2_kit.mapped_zip`: Zipped BAM files containing the output from mapping HiFi Reads to the reference genome, by sample.
- `samples.consensus_mapped.bam.zip`: Zipped BAM files containing the output from mapping consensus FASTA files to the reference genome, by sample.
- `samples.coverage.png.zip`: Zipped per-sample coverage graphs in png format.
- `hifi_reads.fastq.zip`: Zipped file of per-sample trimmed HiFi Reads in FASTQ format.
- `sample_summary.csv`: Sample Summary file in CSV format, with one row per sample.

Options	Description
`--task-option sample_wells_csv`	Specifies a 4-column CSV file used to generate the Plate QC Report, which displays analysis results for each sample in the assay plate. The CSV file **must** contain barcode pairs, Bio Sample name, Plate IDs, and Well IDs. The report is useful for diagnosing sample issues based on plate location. (Default = `None`)
`--task-option min_coverage`	Specifies the minimum read coverage. **Below** this value, the consensus sequence will be set to `N`s and no variants are called. (Default = `4`)
`--task-option min_alt_freq`	Specifies that only variants whose frequency is **greater** than this value are reported. This frequency is determined based on the read depth (DP) and allele read count (AD) information in the VCF output file. We recommend using the default value to properly call the dominant alternative variant while also filtering out potential artifacts. (Default = `0.5`)
`--task-option min_bq`	Specifies that reads with barcode scores **below** this minimum value are **not** included in analysis. (Default = `80`)
`--task-option mimux_overrides`	Specifies additional options to pass to the `mimux` preprocessing tool for trimming and filtering reads by probe sequences. Options should be entered in space-separated format. Available options include: `--max-len`: Specifies the maximum sequence length. (Default = `800`) `--same`: Specifies that only reads with arms sequences from the same probe are used.

Running the SARS-CoV-2 Analysis Application

```
pbcromwell run pb_sars_cov2_kit \

-e <movie.consensusreadset.xml> \

-e $SMRT_ROOT/current/bundles/smrtinub/current/private/pacbio/barcodes/HiFiViral_SARS-
CoV-2_Enrichment_Probes.barcodeset.xml \

-e eid_ref_dataset_2:$SMRT_ROOT/current/bundles/smrtinub/current/private/pacbio/
canneddata/referenceset/SARS-CoV-2/sars_cov2.referenceset.xml

--task-option min_alt_freq=0.5 \
--task-option min_bq=80 \
--task-option mimux_overrides="--max-len=800 --same" \
--task-option sample_wells_csv=None \
--config cromwell.conf \
--nproc 8
```

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 `pbmm2` 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 `pbmm2` 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.

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. 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. 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.

Column	Description
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.
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 enables analysis and functional characterization of transcript isoforms for sequencing data generated on PacBio instruments. The analysis is performed *de novo*, without a reference genome.

Usage

```
isoseq3 <tool>
```

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

Typical workflow

1. If you don't already have CCS Reads, generate them from a subreads BAM file. By default, CCS Analysis will run with Polish=ON (contains QVs).

```
ccs movie.subreads.bam movie.ccs.bam --min-rq 0.9
```

2. Visualize primers, then remove primers and demultiplex:

```
cat primers.fasta
>5p
GCAATGAAGTCGCAGGGTTGGGG
>3p
GTACTCTGCGTTGATACCACTGCTT

lima movie.ccs.bam primers.fasta demux.bam --isoseq
```

See "Demultiplex Barcodes" on page 20 for details on the `lima` tool.

3. Remove noise from FL reads:

```
isoseq3 refine demux.5p--3p.bam primers.fasta flnc.bam --require-polya
```

4. Cluster consensus sequences to generate transcripts. This will generate `unpolished.hq.bam` and `unpolished.hq.fasta.gz` files, which are the high-quality (HQ) transcripts that should be analyzed further. **Note**: HQ transcripts generated from this step do **not** contain Quality Values.

```
isoseq3 cluster flnc.bam unpolished.bam --use-qvs
```

5. (**Optional**) If QVs are desired, run `isoseq3 polish`, which takes significantly longer to complete:

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

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

```
pbmm2 align unpolished.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
```

See "pbmm2" on page 80 for details.

`refine` Tool: Remove polyA and concatemers from full-length (FL) reads and generate full-length non-concatemer (FLNC) transcripts (FL to FLNC).

Usage

```
isoseq refine [options] <ccs.demux.bam|xml> <primer.fasta|xml>
<flnc.bam|xml>
```

Inputs/Outputs	Description
`ccs.demux.bam|xml`	Input demultiplexed CCS Reads BAM or `ConsensusReadSet` XML file.
`primer.fasta|xml`	Input primer FASTA or `BarcodeSet` XML file.
`flnc.bam|xml`	Output flnc BAM or `ConsensusReadSet` XML file.

Preprocessing	Description
`--min-polya-length`	Specifies the minimum poly(A) tail length. (Default = `20`)
`--require-polya`	Requires FL reads to have a poly(A) tail and remove it.

Options	Description
`--help, -h`	Displays help information and exits.
`--version`	Displays program version number and exits.
`--verbose, -v`	Sets the verbosity level.
`-j,--num-threads`	Specifies the number of threads to use; `0` means autodetection. (Default = `0`)
`--log-file`	Writes the log to a file. (Default = `stderr`)
`--log-level`	Specifies the log level; values are `[DEBUG, INFO, WARN, TRACE, FATAL]`. (Default = `WARN`)

`cluster` Tool: Cluster FLNC reads and generate transcripts.

Usage

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

Example

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

Custom BAM Tags

`isoseq3 cluster` 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 commas. Example: 0,1,20;0,3,5
- `im`: ZMW names associated with this isoform.
- `is`: Number of ZMWs associated with this isoform.

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

Options	Description
`--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 Analysis Quality Values; sets `--poa-cov` to `100`.

Options	Description
`--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`)

`polish` Tool: Polish transcripts using Continuous Long Reads subreads.

Usage

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

Custom BAM Tags

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

- `iz`: Maximum number of subreads used for polishing.
- `rq`: Predicted accuracy for polished isoform.

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`)

`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 `polished.bam` file.
`output`	`summary.csv` file.

Options	Description
`--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`)

`collapse` Tool: Collapse transcripts based on genomic mapping.

Usage

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

Examples:

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

Inputs/Outputs	Description
`alignments`	Alignments mapping polished or unpolished transcripts to the reference genome. (BAM or XML file).
`ccs.bam`	Optional input BAM file containing CCS Reads.
`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`)

Options	Description
`-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 Reads, 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.
`--mode-phasing,-p`	Phases variants and cluster haplotypes.

Input Files

- BAM-format files containing CCS Reads. 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 CCS Analysis 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*
A T G	M	41	L	T T G	1	2793	ABC + DDI + TDF + D4T + ZDV
A A A	K	65	R	A G A	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

T A T	Y	181	C	T G T	0.91	2946	NVP + EFV + ETR + RPV
G G A	G	190	A	G C A	1	2947	NVP + EFV + ETR + RPV
A C C	T	215	Y	T A C	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

Drug	Gene	Reference AA	Reference Pos	Sample AA	Sample %
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, 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..."
```

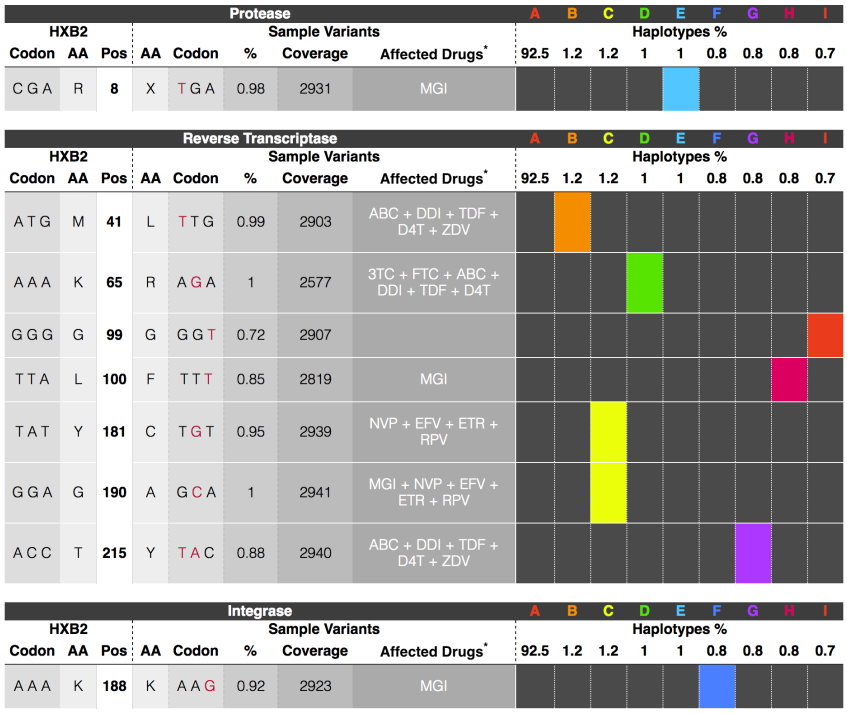
No Target Configuration

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

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

Reverse Transcriptase

HXB2 Codon	AA	Pos	Sample Variants AA	Codon	%	Coverage	Affected Drugs*	Haplotypes % A 92.5	B 1.2	C 1.2	D 1	E 1	F 0.8	G 0.8	H 0.8	I 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									

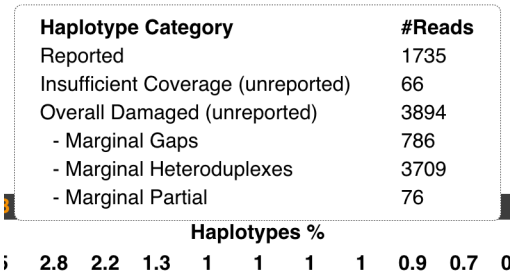
Integrase

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

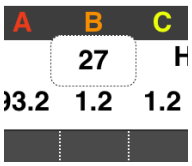
- The row-wise variant calls are "transposed" onto per-column haplotypes. Each haplotype has an ID: `[A-Z]{1}[a-z]?`.
- For each variant, colored boxes in this row mark haplotypes that contain this variant.
- Colored boxes per haplotype/column indicate variants that co-occur. Wild type (no variant) is 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:



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.

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.

Microbial Assembly

The Microbial Assembly application is powered by the IPA HiFi genome assembler and includes the following feature:

- Polishes the contigs with phased reads using `Racon`.

Workflow of the Microbial Assembly Application

The workflow consists of several steps around 2 main stages:

1. Chromosomal stage: Assemble large contigs using IPA, the Hifi Genome Assembly tool.
2. Separate reads that were used for accurate large contigs from all other reads.
3. Plasmid stage: Assemble plasmids (using IPA) from the reads separated in the previous step.
4. De-duplicate plasmids.
5. Collect all contigs into a single FASTA file.
6. Rotate circular contigs.
7. Align the input dataset to the assembled contigs.

The application accepts HiFi XML Data Sets as input, and has an embedded downsampling feature:

- If the genome size and the desired coverage are specified, **both** stages are downsampled, as with the Genome Assembly Application.
- Otherwise, the full coverage is used.

The embedded downsampling feature is **not** applied to the alignment stage – **all** input reads will be aligned against the assembled contigs.

Usage

The Microbial Assembly application is run using the `pbcromwell` run command, with the `pb_assembly_hifi_microbial` parameter to specify the application. See "pbcromwell" on page 74 for details.

To view information on the available Microbial Assembly options, enter:

```
pbcromwell show-workflow-details pb_assembly_hifi_microbial
```

The minimum command needed to run the workflow requires the input and the number of threads. The following example uses 16 threads:

```
pbcromwell run pb_assembly_hifi_microbial -e <input.xml> --nproc 16
```

The following example performs an assembly using an input XML Data Set, and uses all default settings, including 1 CPU:

```
pbcromwell run pb_assembly_hifi_microbial -e <input.consensusreadset.xml>
```

Note: To specify different task options on the command line, consider the following example:

```
pbcromwell run pb_assembly_hifi_microbial \
-e <input.consensusreadset.xml> \
--task-option reads=None \
--task-option ipa2_genome_size=0 \
--task-option ipa2_downsampled_coverage=0 \
--task-option microasm_plasmid_contig_len_max=300000
--task-option ipa2_cleanup_intermediate_files=True \
--task-option dataset_filters="" \
--task-option filter_min_qv=20 \
--nproc 8
```

The default options for this application should work well for any genome type.

As microbes are relatively small, we rarely find much advantage to using more than 4 threads, or more than 2 concurrent jobs.

Microbial Assembly Parameters

Option	Default Value	Description
`-e, --eid_ccs`	NONE	Optional parameter, required if `--task-option reads <input>` is **not** specified. This is a SMRT Link-specific input parameter and supports **only** PacBio `Consensusreadset` XML files as input.
`--task-option reads`	NONE	Optional parameter, required if `-e <input>` is **not** specified. Supports multiple input formats: FASTA, FASTQ, BAM, XML, FOFN and gzipped versions of FASTA/FASTQ.

Option	Default Value	Description
`--task-option ipa2_genome_size`	10M	The approximate number of base-pairs expected in the chromosomal genome. (We assume that any plasmids are a tiny fraction of the total.) Used only for downsampling in the assembly stages (that is, not in polishing). If value `<=0`, then downsampling is off. **Default**: `10M` (10 Mega basepairs). **Note**: `ipa2_genome_size` is currently the **only** option that accepts a metric suffix. Commas and decimals are not accepted anywhere. `ipa2_genome_size` actually accepts a String, which can be an integer followed by one of these metric suffixes: `k/M/G`. For example: `4500k` means “4,500 kilobases” or “4,500,000”. `M` stands for Mega and `G` stands for Giga.
`--task-option ipa2_downsampled_coverage`	100	The maximum coverage after downsampling, assuming the estimated `genome_size` was high enough. (The default `genome_size` was chosen to be larger than any realistic microbe.) If `<=0`, then downsampling is off. **Default**: `100` **Example**: If your genome is 1G in length, and you specify `ipa2_genome_size=2G`, you have over-estimated by 2x. If you also specify `ipa2_downsampled_coverage=100`, your data will be downsampled to 200x coverage, simply because of the over-estimate. The cost of extra coverage is greater runtime. The defaults are usually fine.

`--task-option ipa2_advanced_options_chrom`	See Description Column	A semicolon-separated list of KEY=VALUE pairs. New line characters are not accepted. (These are described later in this document.)
		`config_block_size = 100;`
		`config_seeddb_opt = -k 28 -w 20 --space 0 --use-hpc-seeds-only;`
		`config_ovl_opt = --one-hit-per-target --min-idt 98 --traceback --mask-hp --mask-repeats --trim --trim-window-size 30 --trim-match-frac 0.75`

`--task-option ipa2_advanced_options_plasmid`	See Description Column	A semicolon-separated list of KEY=VALUE pairs. New line characters are not accepted. (These are described later in this document.)
		`con-fig_block_size = 100;`
		`con-fig_ovl_filter_opt = --max-diff 80 --max-cov 100 --min-cov 2 --bestn 10 --min-len 500 --gapFilt --minDepth 4 --idt-stage2 98;`
		`con-fig_ovl_min_len = 500;`
		`con-fig_seeddb_opt = -k 28 -w 20 --space 0 --use-hpc-seeds-only;`
		`config_ovl_opt = --one-hit-per-target --min-idt 98 --min-map-len 500 --min-anchor-span 500 --traceback --mask-hp --mask-repeats --trim --trim-window-size 30 --trim-match-frac 0.75 --smart-hit-per-target --secondary-min-ovl-frac 0.05; con-fig_layout_opt = --allow-circular;`

`--task-option ipa2_cleanup_intermediate_files`	TRUE	Removes intermediate files from the run directory to save space.
`--task-option microasm_plasmid_contig_len_max`	300000	After the chromosomal stage, in task `filter_draft_contigs`, separates long contigs (presumed to be chromosomal) from shorter contigs (to be re-assembled in the plasmid stage). Then, after the plasmid stage, in `dedup_plasmids`, contigs less than this value are ignored. The default value is usually fine.
`--task-option consolidate_aligned_bam`	FALSE	Consolidates Mapped BAMs for IGV.

Option	Default Value	Description
`--task-option dataset_filters`	NONE	**(General pbcromwell option)** A semicolon-separated (**not** comma-separated) list of other filters to add to the Data Set.
`--task-option filter_min_qv`	20	**(General pbcromwell option)** Phred-scale integer QV cutoff for filtering HiFi Reads. The default for all applications (**except** Iso-Seq analysis) is `20` (QV 20), or 99% predicted accuracy.
`--task-option downsample_factor`	0	Downsamples the input Data Set by a given factor. This is applied on the entire input Data Set, and affects both assembly and alignment stages.
`--task-option log_level`	`INFO`	Specifies the logging (verbosity) level for tools which support this feature. Values are: [`TRACE, DEBUG, INFO, WARN, FATAL`]
`--task-option max_nchunks`	40	Specifies the maximum number of chunks per task.
`--task-option tmp_dir`	`/tmp`	Specifies an optional temporary directory for used by several subtools, such as `sort`.

Input Files

- `*.bam` file containing PacBio data.
- `*.fasta` or `*.fastq` file containing PacBio data.
- `*.xml` file containing PacBio data.
- `*.fofn` files with file names of files containing PacBio data.

Output Files

- `final_assembly.fasta`: File containing all assembled contigs, rotated.
- `assembly.rotated.polished.renamed.fsa`: File for NCBI, but otherwise identical to `final_assembly.fasta`; only the headers are changed.

Advanced Parameters

Note: Advanced parameters should be rarely modified. Advanced parameters for the Microbial Assembly workflow are the **same** as those for the Genome Assembly workflow. See "Advanced Parameters" on page 39 for details.

Advanced parameters specified on the command line:

- Are in the form of `key = value` pairs.
- Each pair is separated by a semicolon (`;`) character.
- The full set of advanced parameters is surrounded by **one** set of double quotes.
- The specified value of a parameter **overwrites** the default options for that key **– all** desired options of that parameter must be explicitly listed, not just the ones which should change from the default.
- Setting an empty value **clears** the parameter; it does **not** reset the value back to default.

Example:

```
 --task-option ipa2_advanced_options_chrom="config_seeddb_opt=-k
30;config_block_size=2048"
```

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 here.

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.

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".

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 87.)

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

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]
                   [run,show-workflows,show-workflow-details,configure,submit,get-
job,abort,metadata,show-running,wait]
```

Options	Description
`--output-dir OUTPUT_DIR`	Specifies the output directory for `cromwell` output. (Default = `cromwell_out`)
`--overwrite`	Overwrites the output directory, if it exists. (Default = `False`)
`-i INPUTS, --inputs INPUTS`	Specifies `cromwell` inputs and settings as JSON files. (Default = `None`)
`-e ENTRY_POINTS, --entry ENTRY_POINTS`	Specifies the 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`	Specifies the number of processors per task. (Default = `1`)
`-c MAX_NCHUNKS, --max-nchunks MAX_NCHUNKS`	Specifies the maximum number of chunks per task. (Default = `None`)
`--target-size TARGET_SIZE`	Specifies the target chunk size. (Default = `None`)
`--queue QUEUE`	Specifies the cluster queue to use. (Default = `None`)
`-o OPTIONS, --options OPTIONS`	Specifies additional `cromwell` engine options, as a JSON file. (Default = `None`)
`-t TASK_OPTIONS, --task-option TASK_OPTIONS`	Specifies workflow- or task-level option as `key=value` strings, specific to the application. May be specified multiple times for multiple options. (Default = `[]`)

Options	Description
-b BACKEND, --backend BACKEND	Specifies the backend to use for running tasks. (Default = None)
-r MAX_RETRIES, --maxRetries MAX_RETRIES	Specifies the maximum number of times to retry a failing task. (Default = 1)
--tmp-dir TMP_DIR	Specifies an optional temporary directory for cromwell tasks, which **must** exist on all compute hosts. (Default = None)
--config CONFIG	Specifies a Java configuration file for running cromwell. (Default = None)
--dry-run	Specifies that cromwell is **not** executed, but instead writes out final inputs and then exits. (Default = True)
workflow	Specifies the workflow ID (such as pb_ccs or cromwell.workflows.pb_ccs for PacBio workflows only) or a path to a Workflow Description Language (WDL) source file.

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

Examples:

Show available PacBio-developed workflows:

```
pbcromwell show-workflows
```

Show details for a PacBio workflow:

```
pbcromwell 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)
--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.

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 here.

Workflow Examples:

Run the CCS Analysis:

```
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
```

`pbcromwell show-workflow-details` Command: Display details about a specified PacBio workflow, including input files and task options.

Usage:

```
pbcromwell show-workflow-details [-h] [--inputs-json INPUTS_JSON]
                                 workflow_id
```

Options	Description
`workflow_id`	Specifies the workflow ID, such as `pb_ccs` or `cromwell.workflows.pb_ccs` for PacBio workflows **only**.
`--inputs-json INPUTS_JSON`	Writes a JSON template containing workflow inputs to the specified file. (Default = `None`)

`pbcromwell configure` Command: Generate the Java configuration file used by `cromwell` to define backends and other important engine options that **`cannot`** be set at runtime. You can pass this to
`pbcromwell run` using the `--config` argument.

Usage:

```
pbcromwell configure [-h] [--port PORT]
                         [--local-job-limit LOCAL_JOB_LIMIT]
                         [--jms-job-limit JMS_JOB_LIMIT]
                         [--db-port DB_PORT] [--db-user DB_USER]
                         [--db-password DB_PASSWORD]
                         [--default-backend DEFAULT_BACKEND]
                         [--max-workflows MAX_WORKFLOWS]
                         [--output-file OUTPUT_FILE] [--timeout TIMEOUT]
                         [--cache] [--no-cache]
```

Options	Description
`--port PORT`	Specifies the port that `cromwell` should listen to. (Default = `8000`)
`--local-job-limit LOCAL_JOB_LIMIT`	Specifies the maximum number of local jobs/tasks that can be run at once. (Default = `10`)
`--jms-job-limit JMS_JOB_LIMIT`	Specifies the maximum number of jobs/tasks that can be submitted to the queueing system at one time. (Default = `500`)
`--db-port DB_PORT`	Specifies the database port for `cromwell` to use; if undefined, database configuration is omitted. (Default = `None`)
`--db-user DB_USER`	Specifies the user name tused to connect to Postgres. (Default = `smrtlink_user`)
`--db-password DB_PASSWORD`	Specifies the password used to connect to Postgres.
`--default-backend DEFAULT_BACKEND`	Specifies the default job execution backend. Choices are: `Local`, `SGE`, `Slurm`, `PBS`, or `LSF`. (Default = `Local`)
`--max-workflows MAX_WORKFLOWS`	Specifies the maximum number of workflows that `cromwell` can run at once. (Default = `100`)
`--output-file OUTPUT_FILE`	Specifies the name of the output configuration file. (Default = `cromwell.conf`)

Options	Description
`--timeout TIMEOUT`	Specifies the time to wait for task completion before checking the cluster job status. (Default = `600`)
`--cache`	Enables call caching. (Default = `True`)
`--no-cache`	Disables call caching. (Default = `True`)

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.

pbmarkdup

The `pbmarkdup` tool marks PCR duplicates in CCS Reads Data Sets from amplified libraries. PCR duplicates are different reads that arose from amplifying a single-source molecule. `pbmarkdup` can also optionally remove the duplicate reads.

Note: `pbmarkdup` only works with CCS Reads, **not** with Continuous Long Reads.

`pbmarkdup` uses a reference-free comparison method. Duplicates are identified as pairs of reads that:

1. Have the same length - within 10 bp, **and**
2. Have high percent identity alignments at the molecule ends at >98% identity of the first and last 250 bp.

Clusters are formed from sets of two or more duplicate reads, and a single read is selected as the representative of each cluster. Other reads in the cluster are considered duplicates.

How are duplicates marked?

In FASTA and FASTQ formats, reads from duplicate clusters have annotated names. The following is a FASTA example:

```
>m64013_191117_050515/67104/ccs DUPLICATE=m64013_191117_050515/3802014/ccs DS=2
```

This shows a marked duplicate read `m64013_191117_050515/67104/ccs` that is a duplicate of `m64013_191117_050515/3802014/ccs` in a cluster with 2 reads (`DS` value). Accordingly, the following is the read selected as the representative of the molecule:

```
>m64013_191117_050515/3802014/ccs DS=2
```

In BAM format, duplicate reads are flagged with `0x400`. The read-level tag `ds` provides the number of reads in a cluster (like the `DS` attribute described above for FASTA/FASTQ), and the `du` tag provides the name of the representative read (like the `DUPLICATE` attribute described above for FASTA/FASTQ).

Usage

```
pbmarkdup [options] <INFILE.bam|xml|fa|fq|fofn> <OUTFILE.bam|xml|fa.gz|fq.gz>
```

Options	Description
`-h, --help`	Displays help information and exits.
`--version`	Displays program version number and exits.
`--log-file`	Logs to a file, instead of `stderr`.
`--log-level`	Specifies the log level; values are `[TRACE,DEBUG,INFO,WARN, FATAL]` (Default = `WARN`) `--log-level INFO` produces a summary report such as: LIBRARY READS UNIQUE MOLECULES DUPLICATE READS --- <Unnamed> 25000 24948 (99.8%) 52 (0.2%) SS-lib 496 493 (99.4%) 3 (0.6%) --- TOTAL 25496 25441 (99.8%) 55 (0.2%)
`-j,--num-threads`	Specifies the number of threads to use, `0` means autodetection. (Default = `0`)

Duplicate Marking Options	Description
`--cross-library, -x`	Identifies duplicate reads across sequencing libraries. Libraries are specified in the BAM read group `LB` tag.

Output Options	Description
`-rmdup, -r`	Excludes duplicates from `OUTFILE`. (This is redundant when `--dup-file` is specified.)
`--dup-file FILE`	Stores duplicate reads in an extra file other than `OUTFILE`. The format of this file can be different from the output file.
`--clobber, -f`	Overwrites `OUTFILE` if it exists.

Input Files

CCS Reads from one or multiple movies in any of the following formats:

- PacBio BAM (`.ccs.bam`)

- PacBio dataset (`.dataset.xml`)
- File of File Names (`.fofn`)
- FASTA (`.fasta`, `.fasta.gz`)
- FASTQ (`.fastq`, `.fastq.gz`)

Output Files

CCS Reads with duplicates marked in a format inferred from the file extension:

- PacBio BAM (`.ccs.bam`)
- PacBio dataset (`.dataset.xml`), which also generates a corresponding BAM file.
- FASTA (`.fasta.gz`)
- FASTQ (`.fastq.gz`)

Allowed Input/Output Combinations

Input File	Output BAM	Output Dataset	Output FASTQ	Output FASTA
Input BAM	Allowed	Allowed	Allowed	Allowed
Input Dataset	Allowed	Allowed	Allowed	Allowed
Input FASTQ	Not Allowed	Not Allowed	Allowed	Allowed
Input FASTA	Not Allowed	Not Allowed	Not Allowed	Allowed

Examples

Run on a single movie:

```
pbmarkdup movie.ccs.bam output.bam
```

Run on multiple movies:

```
pbmarkdup movie1.fasta movie2.fasta output.fasta
```

Run on multiple movies and output duplicates in separate file:

```
pbmarkdup movie1.ccs.bam movie2.fastq uniq.fastq --dup-file dups.fasta
```

pbmm2

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

`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`.

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

- `mg`, stores gap-compressed alignment identity, defined as `nM/(nM + nMM + nInsEvents + nDelEvents)`.

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` The option is **not** case-sensitive. (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.)
`--short-sa-cigar`	Populates the `SA` tag with the short cigar representation.

`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.
`-y,--min-gap-comp-id-perc`	Specifies the minimum gap-compressed sequence identity, 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.
`--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`)

Parameter Set Options and Overrides	Description
`-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 Reads 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 are 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.

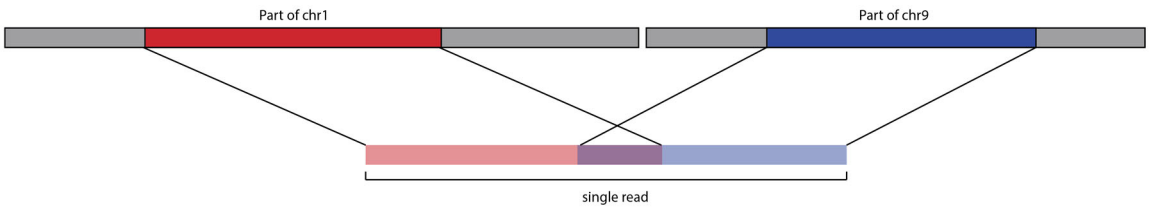
What other special parameters are used implicitly?

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` 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 is 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`.
`--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-run` Command**:** Create a SMRT Link Run Design and optional Auto Analysis jobs from a CSV file. This is equivalent to the Run Design CSV import feature in the SMRT Link UI; the resulting runs can then be edited in the UI or executed on-instrument.

```
pbservice import-run [-h][--host HOST] [--port PORT]
                         [--log-file LOG_FILE]
                         [--log-level INFO]
                         [--debug] [--quiet]
                         [--dry-run]
                         csv_file
```

Required	Description
`csv_file`	Specifies a Run Design CSV-format file.

Option	Description
`--dry-run`	Prints the generated run XML without POSTing to the server.

`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.

Options	Description
`--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).
`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 = 100)
-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)
-y,--min-gap-comp-id-perc	Ignores alignments with gap-compressed sequence identity < N%. (Default = 70)

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)
-r,--region	Limits discovery to this reference region: CHR|CHR:START-END
-t,--types	Calls these SV types: "DEL", "INS", "INV", "DUP", "BND", "CNV". (Default = "DEL,INS,INV,DUP,BND")
-m,--min-sv-length	Ignores variants with length < N bp. (Default = 20)
--max-ins-length	Ignores insertions with length > N bp. (Default = 15K)
--max-dup-length	Ignores duplications with length > N bp. (Default = 1M)
--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 = 3)
-O, --call-min-reads-one-sample	Ignores calls supported by < N reads in every sample. (Default = 3)
-S, --call-min-reads-per-strand-all-samples	Ignores calls supported by < N reads per strand total across samples. (Default = 1)
-B,--call-min-bnd-reads-all-samples	Ignores BND calls supported by < N reads total across samples. (Default = 2)
-P, --call-min-read-perc-one-sample	Ignores calls supported by < P% of reads in every sample. (Default = 20)
--preserve-non-acgt	Preserves non-ACGT in REF allele instead of replacing with N. (Default = false)
--hifi, --ccs	Uses options optimized for HiFi Reads: -S 0 -P 10. (Default = false)
--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.)
--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 Reads BAM Input:

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

CCS Reads 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 Analysis 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 here 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,ContigSet,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`)

Options	Description
`--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
```

runqc-reports

The `runqc-reports` tool generates up to five different Run QC reports, depending on Data Set type: Raw Data, Adapters, Loading, Control, and CCS Reads. Generating a complete set of reports requires the presence of an `sts.xml` resource in the Data Set, but either the CCS Analysis report (or a fallback Subreads report) will **always** be generated. All report JSON and plot PNG files are written to the current working directory, unless otherwise specified.

Usage

```
runqc-reports [-h] [--version] [--log-file LOG_FILE]
              [--log-level {DEBUG,INFO,WARNING,ERROR,CRITICAL}]
              [| --debug | --quiet | -v]
              [-o OUTPUT_DIR]
              dataset_xml
```

Required	Description
`dataset_xml`	Input SubreadSet or ConsensusReadSet XML, which **must** contain an `sts.xml` resource for the full Run QC report set to be generated.
`-o OUTPUT_DIR`	Output report directory. (`Default` = Current working directory)

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 = `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`)
`--verbose, -v`	Sets the verbosity level. (Default = `None`)

Examples

```
runqc-reports moviename.subreadset.xml

runqc-reports moviename.consensusreadset.xml
```

summarize Modifications

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

Usage

```
summarizeModifications [-h] [--version]
                       [--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.
`--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

Note: To print information about a specific PacBio workflow, including input files and task options, use the `pbcromwell show-workflow-details` command, which is available for **all** applications. **Example**:

```
pbcromwell show-workflow-details pb_hgap4
pbcromwell show-workflow-details cromwell.workflows.pb_hgap4
```

(The prefix `cromwell.workflows` is optional.)

Assembly (HGAP 4) (CLR Reads Only)

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 (CLR Reads Only)

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) (CLR Reads Only)

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`
<moviename>.hifi.reads.bam file	`ccs_bam_out`
Consensus Sequences	`pb_ccs.ccsxml`
CCS Analysis Statistics	`pb_ccs.report_ccs`
All Reads (BAM)	`reads_bam`
<moviename>.hifi.reads.fasta	`ccs_fasta_out`
<moviename>.hifi.reads.fastq	`ccs_fastq_out`

CCS with Demultiplexing (CLR Reads Only)

Analysis Application Name: `cromwell.workflows.pb_ccs_demux`

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
FASTQ file	`ccs_fastq_out`
FASTA file	`ccs_fasta_out`
BAM file	`ccs_bam_out`
Consensus Sequences	`pb_ccs.ccsxml`
CCS Analysis Statistics	`pb_ccs.report_ccs`
Barcode Report Details	`pb_demux_ccs.summary_csv`
Demultiplexed Data Sets	`pb_demux_ccs.demuxed_files_datastore`
Unbarcoded Reads	`pb_demux_ccs.unbarcoded`

CCS with Mapping (CLR Reads Only)

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 Analysis 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 (CLR Reads Only)

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 (CLR Reads Only)

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 (HiFi Reads 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.demuxed_files_datastore`
Unbarcoded Reads	`pb_demux_ccs.unbarcoded`

Export Reads (HiFi Reads Only)

Analysis Application Name: `cromwell.workflows.pb_export_ccs`

Entry Points

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

Key Output Files

File Name	Datastore SourceId
`<moviename>.hifi_reads.fastq.gz`	`ccs_fastq_out`
`<moviename>.hifi_reads.fasta.gz`	`ccs_fasta_out`
`<moviename>.hifi_reads.bam.gz`	`ccs_bam_out`

Note: If users select a lower cutoff Phred QV value, the string `hifi` is replaced by the QV value in the file names.
Example: `<moviename>.q10.fastq.gz`.

Genome Assembly (HiFi Reads Only)

Analysis Application Name: `cromwell.workflows.pb_assembly_hifi`

Entry Points

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

Key Output Files

File Name	Datastore SourceId
Final Polished Assembly, Primary Contigs	pb_assembly_hifi.final_primary_contigs_fasta
Final Polished Assembly, Haplotigs	pb_assembly_hifi.final_haplotigs_fasta
List of Circular Contigs	pb_assembly_hifi.circular_contigs
Summary Report	pb_assembly_hifi.report_polished_assembly

HiFiViral SARS-CoV-2 Analysis (HiFi Reads Only)

Analysis Application Name: cromwell.workflows.pb_sars_cov2_kit

Entry Point

```
:id: eid_ccs (HiFi Reads, demultiplexed as separate BAM files)
:name: Entry eid_ccs
:fileTypeId: PacBio.DataSet.ConsensusReadSet

:id: eid_ref_dataset_2 (Reference Genome)
:name: Entry eid_ref_dataset
:fileTypeId: PacBio.DataSet.ReferenceSet

:id: eid_barcode (Probe Sequences)
:name: Entry eid_barcode
:fileTypeId: PacBio.DataSet.BarcodeSet
```

Key Output Files

File Name	Datastore SourceId
All Samples, Probe Counts TSV	pb_sars_cov2_kit.probe_counts_zip
All Samples, Raw Variant Calls VCF	pb_sars_cov2_kit.raw_vcf_zip
All Samples, Variant Call VCF	pb_sars_cov2_kit.vcf_zip
All Samples, Variant Calls CSV	pb_sars_cov2_kit.variants_csv
All Samples, Consensus Sequence FASTA	pb_sars_cov2_kit.fasta_zip
All Samples, Consensus Sequence By Fragments FASTA	pb_sars_cov2_kit.frag_fasta_zip
All Samples, Aligned Reads BAM	pb_sars_cov2_kit.mapped_zip
All Samples, Consensus Sequence Aligned BAM	pb_sars_cov2_kit.aligned_frag_zip
All Samples, Trimmed HiFi Reads FASTQ	pb_sars_cov2_kit.trimmed_zip
Failed Sample Info	pb_sars_cov2_kit.sample_failures_csv
Failed Sample Analysis Logs	pb_sars_cov2_kit.errors_zip
Demultiplex Summaries	pb_sars_cov2_kit.lima_summary_zip
Sample Summary Table CSV	pb_sars_cov2_kit.summary_csv
All Samples, Genome Coverage Plots	pb_sars_cov2_kit.coverage_png_zip

Iso-Seq Analysis (HiFi Reads Only)

Analysis Application Name: `cromwell.workflows.pb_isoseq3_ccsonly`

Entry Points

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

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

:id: eid_ref_dataset
:name: Reference (Optional)
: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`
Collapsed Isoforms Abundance TXT (Files are numbered consecutively, 1 for each barcoded sample.)	`pb_isoseq3_ccsonly.collapse_abundance` Separate clusters, one per barcoded sample.
Collapsed Isoforms Abundance TXT (Files are numbered consecutively, 1 for each barcoded sample.)	`pb_isoseq3_ccsonly.collapse_abundance` Pooled clusters, one per barcoded sample.
High-Quality Transcripts	`pb_isoseq3_ccsonly.hq_fastq`
Low-Quality Transcripts	`pb_isoseq3_ccsonly.lq_fastq`
High-Quality Transcripts Counts (Files are numbered consecutively, 1 for each barcoded sample.)	`pb_isoseq3_ccsonly.barcode_overview_report` Separate clusters, one per barcoded sample.
High-Quality Transcripts Counts (Files are numbered consecutively, 1 for each barcoded sample.)	`pb_isoseq3_ccsonly.barcode_overview_report` Pooled clusters, one per barcoded sample.
CCS Reads FASTQ	`pb_isoseq3_ccsonly.ccs_fastq_zip`
Full-length CCS Reads	`pb_isoseq3._ccsonly.flnc_bam`
Polished Report	`pb_isoseq3._ccsonly.polish_report_csv`
Cluster Report	`pb_isoseq3._ccsonly.report_isoseq`

Long Amplicon Analysis (LAA) (CLR Reads Only)

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 (CLR Reads Only)

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

Mapping (HiFi Reads Only)

Analysis Application Name: cromwell.workflows.pb_ccs_subreads

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
Mapped reads	pb_align_ccs.mapped
Coverage summary	pb_align_ccs.coverage_gff

Mark PCR Duplicates (HiFi Reads Only)

Analysis Application Name:
cromwell.workflows.pb_mark_duplicates

Entry Points

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

Key Output Files

File Name	Datastore SourceId
PCR Duplicates	pb_mark_duplicates.duplicates
Deduplicated reads	pb_mark_duplicates.deduplicated In the SMRT Link UI, this displays as <ORIGINAL_DATASET_NAME> (deduplicated).

Microbial Assembly (CLR Reads Only)

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/fastq
Draft Assembly	pb_assembly_microbial.draft_assembly
Draft Assembly Index	pb_assembly_microbial.draft_assembly_fai
Final Assembly	pb_assembly_microbial.ncbi_fasta
Mapped BAM	pb_assembly_microbial.mapped
List of Circular Contigs	pb_assembly_microbial.circular_list
Coverage Summary	pb_assembly_microbial.coverage_gff
Coverage Report	pb_assembly_microbial.report_coverage
Mapping Statistics Report	pb_assembly_microbial.report_mapping_stats
Preassembly Report	pb_assembly_microbial.report_preassembly
Polished Assembly Report	pb_assembly_microbial.report_polished_assembly

Microbial Assembly (HiFi Reads Only)

Analysis Application Name:

cromwell.workflows.pb_assembly_hifi_microbial

Entry Points

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

id: reads (only for debugging)
```

Key Output Files

File Name	Datastore SourceId
Final Polished Assembly	pb_assembly_hifi_microbial.assembly_fasta
Final Polished Assembly Index	pb_assembly_hifi_microbial.assembly_fasta.fai

File Name	Datastore SourceId
Final Polished Assembly for NCBI	pb_assembly_hifi_microbial.ncbi_fasta
Mapped BAM	pb_assembly_hifi_microbial.mapped
List of Circular Contigs	pb_assembly_hifi_microbial.circular_list
Coverage Summary	pb_assembly_hifi_microbial.coverage_gff
Coverage Report	pb_assembly_hifi_microbial.report_coverage
Mapping Statistics Report	pb_assembly_hifi_microbial.report_mapping_stats
Mapped BAM Datastore	pb_assembly_hifi_microbial.mapped_bam_datastore
Polished Assembly Report	pb_assembly_hifi_microbial.report_polished_assembly

Minor Variants Analysis (HiFi Reads Only)

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

Site Acceptance Test (SAT) (CLR Reads Only)

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

File Name	Datastore SourceId
Coverage Summary	pb_sat.coverage_gff
Consensus Sequences	pb_sat.consensus_fasta

Structural Variant Calling (CLR Reads Only)

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 (HiFi Reads 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 (Bio Sample Name)	pb_sv_ccs.alignments_by_sample_datastore

Trim gDNA Amplification Adapters (HiFi Reads Only)

Analysis Application Name: cromwell.workflows.pb_trim_adapters

Entry Points

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

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

Note: The barcodes need to be a single primer sequence.

Key Output Files

File Name	Datastore SourceId
Reads Missing Adapters	`pb_trim_adapters.unbarcoded`
PCR Adapter Data CSV	`pb_trim_adapters.summary_csv`
Trimmed reads	`pb_trim_adapters.trimmed` In the SMRT Link UI, this displays as `<ORIGINAL_DATASET_NAME> (trimmed)`.

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.

purge_dups

- Removes haplotigs and contig overlaps in a *de novo* assembly based on read depth.
- See https://github.com/dfguan/purge_dups 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

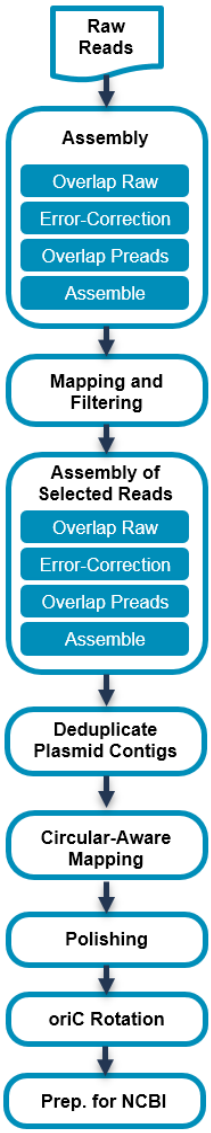
- A set of programs for interacting with high-throughput sequencing data in SAM/BAM/VCF formats.
- See http://www.htslib.org/ for details.

Appendix C - Microbial Assembly Advanced Options (Continuous Long Reads Only)

Use this application to generate *de novo* assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb, using Continuous Long Read data as input.

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 Continuous Long Read 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.

Both stages use an automated random subsampling process to reduce the input Data Set for assembly (by default to 100x). Note that the subsampling is **only** applied to the contig construction process, while the polishing stage of the workflow still uses the **full** input Data Set.

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
remove_temp_data = 1

stage1.length_cutoff = -1
stage1.block_size = 1024
stage1.subsample_coverage = 100
stage1.subsample_random_seed = 12345
stage1.use_median_filter = 1
stage1.autocomp_max_cov = 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 = 400
stage2.subsample_coverage = 100
stage2.subsample_random_seed = 12345
stage2.use_median_filter = 1
stage2.autocomp_max_cov = 0
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 are used as seeds in the draft assembly, and subsequently error-corrected. `-1` means this is calculated automatically so that the total number of seed bases equals (Genome Length x Coverage). `0` means **all** reads in the input Data Set are 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.subsample_coverage`	100	If the input Data Set is large, it will automatically be randomly subsampled to the desired coverage specified by this parameter. The subsampling here is applied **only** to the assembly process, while the polishing stage will still use the full input Data Set. The specified `subsample_coverage` value should be larger than the `coverage` parameter used for seed selection. The difference between these two parameters is that `subsample_coverage` selects reads randomly, while `coverage` picks the longest reads. If `subsample_coverage` is set to `<=0`, subsampling is deactivated.
`stage1.subsample_random_seed`	12345	The value used to seed the random number generator for the subsampling process. Value greater than `0` specifies a fixed seed which allows reproducibility, while a value `<= 0` should produce a different ordering on every run.
`stage1.use_median_filter`	1	The median filter selects one subread per ZMW – the median length subread. `1` enables the filter; `0` deactivates it. It is highly recommended to use the median filter.
`stage1.autocomp_max_cov`	1	If enabled, the maximum allowed overlap coverage at either the 5' or the 3' end of every read is automatically determined based on the statistics computed from the overlap piles. This value is appended to the `ovl_filter_opt` value internally, and supersedes the manually specified values of the `--max-cov` and `--max-diff` parameters. These parameters are used to determine potential repeats and filter out those reads before the string graph is constructed. `1` enables this option, and `0` turns it off.
`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 passed directly to `Raptor`. 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 passed directly to `Raptor`. For details on `Raptor` options, use `raptor -h`.

Advanced Parameters	Default Value	Description
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. Overlaps are formed in the process of alignment, 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.
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 tangles 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. Alternatively, using the autocompute_max_cov option can automatically estimate the value of this parameter, which can improve contiguity (for example, in cases when the input genome size or the seed coverage were overestimated). --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. If the autocompute_max_cov option is used, then the same computed value is supplied to this parameter as well. --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.

Advanced Parameters	Default Value	Description
stage2.length_cutoff	0	Only reads as long as this value are used as seeds in the draft assembly, and subsequently error-corrected. -1 means this is calculated automatically so that the total number of seed bases equals (Genome Length x Coverage). 0 means **all** reads in the input Data Set are used for error-correction.
stage2.block_size	400	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.subsample_coverage	100	If the input Data Set is large, it will automatically be randomly subsampled to the desired coverage specified by this parameter. The subsampling here is applied **only** to the assembly process, while the polishing stage will still use the full input Data Set. The specified subsample_coverage value should be larger than the coverage parameter used for seed selection. The difference between these two parameters is that subsample_coverage selects reads randomly, while coverage picks the longest reads. If subsample_coverage is set to <=0, subsampling is deactivated.
stage2.subsample_random_seed	12345	The value used to seed the random number generator for the subsampling process. Value greater than 0 specifies a fixed seed which allows reproducibility, while a value <= 0 should produce a different ordering on every run.
stage2.use_median_filter	1	The median filter selects one subread per ZMW – the median length subread. 1 enables the filter; 0 deactivates it. It is highly recommended to use the median filter.
stage2.autocomp_max_cov	0	If enabled, the maximum allowed overlap coverage at either the 5' or the 3' end of every read is automatically determined based on the statistics computed from the overlap piles. This value is appended to the ovl_filter_opt value internally, and supersedes the manually specified values of the --max-cov and --max-diff parameters. These parameters are used to determine potential repeats and filter out those reads before the string graph is constructed. 1 enables this option, and 0 turns it off.
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 passed directly to Raptor. 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_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 passed directly to Raptor. 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_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. 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.
stage2.ovl_filter_opt	--max-diff 10000 --max-cov 10000 --min-cov 1 --bestn 20 --min-len 498 --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 tangles 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. Alternatively, using the autocompute_max_cov option can automatically estimate the value of this parameter, which can improve contiguity (for example, in cases when the input genome size or the seed coverage were overestimated). --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. If the autocompute_max_cov option is used, then the same computed value is supplied to this parameter as well. --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.

Advanced Parameters	Default Value	Description
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	The 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 (such as 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 is filtered out and reassembled in the secondary assembly stage. This is performed 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).
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.
remove_temp_data	1	Removes intermediate data once they are no longer needed. This includes the mapped BAM files from the “Mapping and filtering” stage of the workflow. Enabled if set to 1, otherwise this option is disabled.

Appendix D - Microbial Assembly Advanced Options (HiFi Reads Only)

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

The HiFi 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 HiFi Read data (BAM format) as input.

The workflow consists of two assembly stages: **chromosomal** and **plasmid**.

Chromosonal Stage: Intended for contig assembly of large sequences. This stage uses more stringent filtering (using advanced options) to produce contiguous assemblies of complex regions, but it may miss small sequences in the input sample (such as plasmids.)

Plasmid Stage: Intended for a fine-grained assembly. This stage assembles **only** the unmapped and poorly mapped reads. It also relaxes the overlapping parameters, using advanced options.

Both stages use an automated random subsampling process to reduce the input Data Set for assembly (by default to 100x). Note that the subsampling is **only** applied to the contig construction process, while the alignment and reports stages of the workflow still uses the **full** input Data Set.

Available options for the two assembly stages are **identical**. The only differences are:

1. **Chromosonal** stage parameters are prefixed with: `ipa2_advanced_options_chrom`.
2. **Plasmid** stage parameters are prefixed with: `ipa2_advanced_options_plasmid`.

The same sub-options are available to each stage, but the defaults are very different. The current defaults are:

```
ipa2_advanced_options_chrom =

"config_block_size = 100; config_seeddb_opt = -k 28 -w 20 --space 0 --use-hpc-seeds-
only; config_ovl_opt = --one-hit-per-target --min-idt 98 --traceback --mask-hp --mask-
repeats --trim --trim-window-size 30 --trim-match-frac 0.75;"

ipa2_advanced_options_plasmid =
```

```
"config_block_size = 100; config_ovl_filter_opt = --max-diff 80 --max-cov 100 --min-cov
2 --bestn 10 --min-len 500 --gapFilt --minDepth 4 --idt-stage2 98; config_ovl_min_len =
500; config_seeddb_opt = -k 28 -w 20 --space 0 --use-hpc-seeds-only; config_ovl_opt = -
-one-hit-per-target --min-idt 98 --min-map-len 500 --min-anchor-span 500 --traceback --
mask-hp --mask-repeats --trim --trim-window-size 30 --trim-match-frac 0.75 --smart-hit-
per-target --secondary-min-ovl-frac 0.05; config_layout_opt = --allow-circular;"
```

Options are separated by **semicolons**; within each option, parameters are separated by **spaces**.

Users should not need to modify any of default options. If the defaults are modified, workflow behavior could be very different.

Note: The options available in `ipa2_advanced_options_*` are **exactly** the same as the `config_*` options available for the **Genome Assembly** tool. See "Genome Assembly Parameters Input Files" on page 38 for details.

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