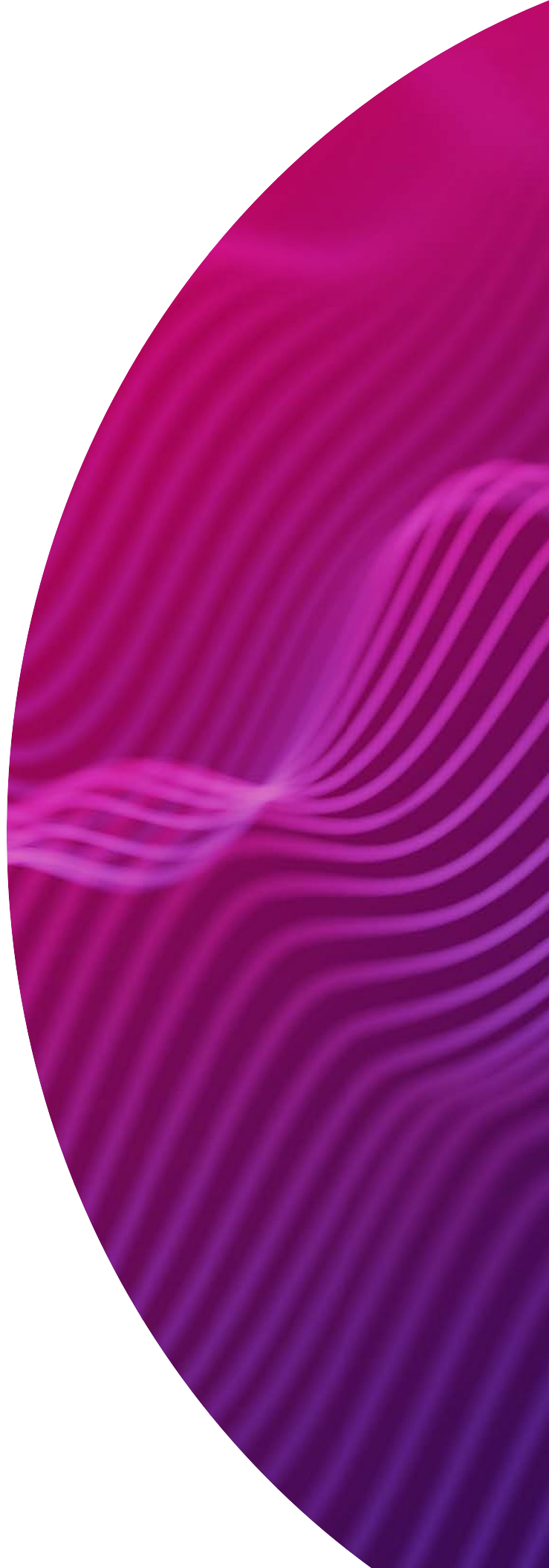




# SMRT<sup>®</sup> Tools reference guide (v11.1)



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## Introduction

This document describes the command-line tools included with SMRT® Link v11.1. 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 guide (v11.1)**.

- **Note:** SMRT Link v11.1 is for use with Sequel® II systems and Sequel IIe systems **only**.
- 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 v11.1 supports all chemistry versions for Sequel II systems and Sequel IIe systems.

## PacBio command-line tools

Following is information on the PacBio-supplied command-line tools included in the installation. For information on third-party tools installed, see [“Appendix B - Third party command-line tools” on page 130](#).

Tool	Description
bam2fasta/ bam2fastq	Converts PacBio® BAM files into gzipped FASTA and FASTQ files. See <a href="#">“bam2fasta/bam2fastq” on page 4</a> .
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 <a href="#">“bamsieve” on page 5</a> .
ccs	Calculates consensus sequences from multiple “passes” around a circularized single DNA molecule (SMRTbell® template). See <a href="#">“ccs” on page 8</a> .
dataset	Creates, opens, manipulates and writes Data Set XML files. See <a href="#">“dataset” on page 17</a> .

Tool	Description
Demultiplex Barcodes	Identifies barcode sequences in PacBio single-molecule sequencing data. See <a href="#">“Demultiplex Barcodes” on page 24.</a>
export-datasets	Takes one or more PacBio dataset XML files and packages all contents into a single ZIP archive. See <a href="#">“export-datasets” on page 36.</a>
export-job	Takes one SMRT Link analysis job and packages all contents into a single ZIP archive. See <a href="#">“export-job” on page 37.</a>
gcpp	Variant-calling tool which provides several variant-calling algorithms for PacBio sequencing data. See <a href="#">“gcpp” on page 39.</a>
Genome Assembly	Generates <i>de novo</i> assemblies using HiFi reads. See <a href="#">“Genome Assembly” on page 42.</a>
HiFiViral SARS-CoV-2 Analysis	Analyzes multiplexed viral surveillance samples for SARS-CoV-2. See <a href="#">“HiFiViral SARS-CoV-2 Analysis” on page 50.</a>
ipdSummary	Detects DNA base-modifications from kinetic signatures. See <a href="#">“ipdSummary” on page 53.</a>
isoseq3	Characterizes full-length transcripts and generates full-length transcript isoforms, eliminating the need for computational reconstruction. See <a href="#">“isoseq3” on page 57.</a>
juliet	A general-purpose minor variant caller that identifies and phases minor single nucleotide substitution variants in complex populations. See <a href="#">“juliet” on page 66.</a>
Microbial Genome Analysis	Generates <i>de novo</i> assemblies of small prokaryotic genomes between 1.9-10 Mb and companion plasmids between 2 – 220 kb and performs base modification detection, using HiFi reads. See <a href="#">“Microbial Genome Analysis” on page 74.</a>
motifMaker	Identifies motifs associated with DNA modifications in prokaryotic genomes. See <a href="#">“motifMaker” on page 78.</a>
pbchromwell	PacBio’s wrapper for the <code>cromwell</code> scientific workflow engine used to power SMRT Link. For details on how to use <code>pbchromwell</code> to run workflows, see <a href="#">“pbchromwell” on page 80.</a>
pbindex	Creates an index file that enables random access to PacBio-specific data in BAM files. See <a href="#">“pbindex” on page 85.</a>
pbmarkdup	Marks or removes duplicates reads from CCS reads. See <a href="#">“pbmarkdup” on page 86.</a>
pbmm2	Aligns PacBio reads to reference sequences; a SMRT wrapper for <code>minimap2</code> . See <a href="#">“pbmm2” on page 89.</a>
pbservice	Performs a variety of useful tasks within SMRT Link. See <a href="#">“pbservice” on page 96.</a>
pbsv	Structural variant caller for PacBio reads. See <a href="#">“pbsv” on page 101.</a>
pbvalidate	Validates that files produced by PacBio software are compliant with PacBio’s own internal specifications. See <a href="#">“pbvalidate” on page 105.</a>
pigeon	Classifies and filters full-length transcript isoforms into categories against a reference annotation. See <a href="#">“pigeon” on page 108.</a>
primrose	Performs motif-calling to detect 5mC CpG sites in HiFi BAM files. See <a href="#">“primrose” on page 114.</a>
runqc-reports	Generates Run QC reports. See <a href="#">“runqc-reports” on page 115.</a>
skera	Splits arrayed HiFi reads at adapter positions, generating read-segments. See <a href="#">“skera” on page 116.</a>

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<b>Tool</b>	<b>Description</b>
summarizeModifications	Generates a GFF summary file from the output of base modification analysis combined with the coverage summary GFF generated by resequencing pipelines. See <a href="#">“summarize Modifications” on page 120</a> .

## **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>
```

<b>Options</b>	<b>Description</b>
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>-o, --output</code>	Specifies the prefix of the output file names. <code>-</code> implies streaming. <b>Note:</b> Streaming is <b>not</b> supported with compression or with the <code>split_barcodes</code> option.
<code>-c</code>	Specifies the Gzip compression level. Values are <code>[1, 2, 3, 4, 5, 6, 7, 8, 9]</code> . (Default = 1)
<code>-u</code>	Specifies that the output FASTA/FASTQ files are <b>not</b> compressed. <code>.gz</code> is not added to the output file names, and <code>-c</code> settings are ignored.
<code>--split-barcodes</code>	Splits the output into multiple FASTA/FASTQ files, by barcode pairs. <b>Note:</b> The <code>bam2fasta/bam2fastq</code> tools inspect the <code>bc</code> tag in the BAM file to determine the 0-based barcode indices from their respective positions in the barcode FASTA file.
<code>-p, --seqid-prefix</code>	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
<code>input_bam</code>	The name of the input BAM file or Data Set from which reads will be read.
<code>output_bam</code>	The name of the output BAM file or Data Set where filtered reads will be written to. (Default = None)

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file LOG_FILE</code>	Writes the log to file. (Default = None, writes to stdout.)
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL]. (Default = WARNING)
<code>--debug</code>	Alias for setting the log level to DEBUG. (Default = False)
<code>--quiet</code>	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
<code>-v, --verbose</code>	Sets the verbosity level. (Default = NONE)
<code>--show-zmws</code>	Prints a list of ZMWs and exits. (Default = False)
<code>--include INCLUDE LIST</code>	Specifies the ZMWs to <b>include</b> 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)

Options	Description
<code>--exclude EXCLUDE LIST</code>	Specifies the ZMWs to <b>exclude</b> 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)
<code>--percentage PERCENTAGE</code>	Specifies a percentage of a SMRT® Cell to recover (Range = 1-100) rather than a specific list of reads. (Default = NONE)
<code>-n COUNT, --count COUNT</code>	Specifies a specific number of ZMWs picked at random to recover. (Default = NONE)
<code>-s SEED, --seed SEED</code>	Specifies a random seed for selecting a percentage of reads. (Default = NONE)
<code>--ignore-metadata</code>	Discard the input Data Set metadata. (Default = False)
<code>--barcodes</code>	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 zms.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 zms.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
```

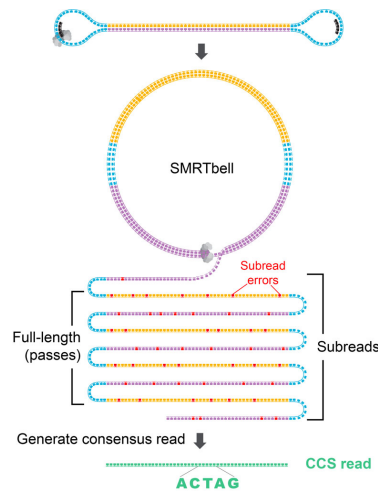
### Splitting a Data Set into two halves:

```
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  $\pm 2$  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:a773clf2 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.
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 <a href="#">"Interpreting QUAL values" on page 13</a> .
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. ( <b>Optional</b> : Depends on barcoded inputs.)

Field	Description
np Tag	The number of full passes that went into the subread. ( <b>Optional</b> : Depends on barcoded inputs.)
rq Tag	The predicted read quality.
zm Tag	The ZMW hole number.
ma Tag	Bitmask storing whether a SMRTbell adapter is missing on either side of the molecule that produced a CCS read. 0 indicates that neither end has a confirmed missing adapter.
ac Tag	An array containing the counts of detected and missing SMRTbell adapters on either side of the molecule that produced a CCS read: <ul style="list-style-type: none"> <li>• Detected adapters on left/start</li> <li>• Missing adapters on left/start</li> <li>• Detected adapters on right/end</li> <li>• Missing adapter on right/end</li> </ul>

## Usage

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

## Example

```
ccs --all myData.subreads.bam myResult.bam
```

Required	Description
Input File Name	The name of a single <code>subreads.bam</code> or a <code>subreadset.xml</code> file to be processed. ( <b>Example</b> = <code>myData.subreads.bam</code> )
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 <code>.xml</code> formats. ( <b>Example</b> = <code>myResult.bam</code> )

Options	Description
<code>--version</code>	Prints the version number.
<code>--report-file</code>	Contains a result tally of the outcomes for all ZMWs that were processed. If <b>no</b> file name is given, the report is output to the file <code>ccs_report.txt</code> . 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.
<code>--min-snr</code>	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 <code>SNR &lt; 2.5</code> is typically considered lower quality. (Default = <code>2.5</code> )
<code>--min-length</code>	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 = <code>10</code> )
<code>--max-length</code>	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 = <code>50000</code> )
<code>--min-passes</code>	Specifies the minimum number of passes for a ZMW to be emitted. This is the number of full passes. Full passes <b>must</b> have an adapter hit before and after the insert sequence and so do <b>not</b> include any partial passes at the start and end of the sequencing reaction. It is computed as the number of passes made across all windows. (Default = <code>3</code> )

Options	Description
<code>--min-rq</code>	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 <code>0.99</code> indicates that only reads expected to be 99% accurate are emitted. (Default = <code>0.99</code> )
<code>--num-threads</code>	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.
<code>--log-file</code>	The name of a log file to use. If none is given, the logging information is printed to <code>STDERR</code> . ( <b>Example:</b> <code>mylog.txt</code> )
<code>--log-level</code>	Specifies verbosity of log data to produce. By setting <code>--logLevel=DEBUG</code> , you can obtain detailed information on what ZMWs were dropped during processing, as well as any errors which may have appeared. (Default = <code>INFO</code> )
<code>--skip-polish</code>	After constructing the draft consensus, do <b>not</b> proceed with the polishing steps. This is significantly faster, but generates less accurate data with no <code>RQ</code> or <code>QUAL</code> values associated with each base.
<code>--by-strand</code>	Separately generates a consensus sequence from the forward and reverse strands. Useful for identifying heteroduplexes formed during sample preparation.
<code>--chunk</code>	Operates on a single chunk. Format <code>i/N</code> , where <code>i</code> in <code>[1,N]</code> . Examples: <code>3/24</code> or <code>9/9</code> .
<code>--max-chunks</code>	Determines the maximum number of chunks, given an input file.
<code>--model-path</code>	Specifies the path to a model file or directory containing model files.
<code>--model-spec</code>	Specifies the name of the chemistry or model to use, overriding the default selection.
<code>--all</code>	<p>Generates <b>one</b> representative sequence per ZMW, irrespective of quality and passes. <code>--min-passes 0 --min-rq 0 --max-length 0</code> are set implicitly and <b>cannot</b> be changed; <code>--all</code> also deactivates the maximum draft length filter. Filtering has to be performed downstream.</p> <p>The <code>ccs --all</code> option changes the workflow as follows:</p> <ol style="list-style-type: none"> <li>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 <code>--all-kinetics</code>, and do not polish.</li> <li>2. Only polish ZMWs with at least two full-length subreads mapping back to the draft. Otherwise, set predicted accuracy <code>rq</code> tag to <code>-1</code> to indicate that the predicted accuracy was not calculated, and populate per-base QVs with <code>+</code> (QV 10) the approximate raw accuracy. Kinetic information is <b>not</b> available for unpolished drafts.</li> <li>3. Instead of using an unpolished draft without kinetic information as a representative consensus sequence, if <code>--subread-fallback</code> is used, fall back to a representative subread with kinetic information.</li> </ol> <p>How is <code>--all</code> different from explicitly setting <code>--min-passes 0 --min-rq 0</code>?</p> <ul style="list-style-type: none"> <li>• Setting <code>--min-passes 0 --min-rq 0</code> is a brute-force combination that polishes <b>every</b> ZMW, even those that only have one partial subread, with polishing making no difference. In contrast, <code>--all</code> is a bit smarter and only polishes ZMWs with at least one full-length subread and one additional partial subread.</li> </ul>

Options	Description
<code>--hifi-kinetics</code>	<p>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 <b>not</b> re-orient these fields.</p> <p>Base modifications can be inferred from per-base pulse width (PW) and inter-pulse duration (IPD) kinetics.</p> <p>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.</p> <p>To facilitate the use of HiFi reads with base modifications workflows, we added an executable in pbbam called <code>ccs-kinetics-bystrandify</code> which creates a pseudo <code>--by-strand</code> BAM with corresponding <code>pw</code> and <code>ip</code> tags that imitates a normal, unaligned subreads BAM.</p>
<code>--all-kinetics</code>	Adds kinetic information for <b>all</b> ZMWs, except for unpolished draft consensus.
<code>--subread-fallback</code>	When combined with <code>--all</code> , uses a subread instead of a draft as representative consensus.
<code>--suppress-reports</code>	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
ref: AC-GGGTATA

*
ccs: 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.
CCS analysis did not converge	None	The consensus sequence did not converge after the maximum number of allowed rounds of polishing.



ZMW results	Parameters affecting results	Description
CCS read below minimum predicted accuracy	<code>--min-rq</code>	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, loadmetadata, copyto, absolutize, relativize}

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>&lt;Command&gt; -h</code>	Displays help for a specific command.
<code>-v, --version</code>	Displays program version number and exits.
<code>--log-file LOG_FILE</code>	Writes the log to file. (Default = None, writes to stdout.)
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL]. (Default = INFO)
<code>--debug</code>	Alias for setting the log level to DEBUG. (Default = False)
<code>--quiet</code>	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
<code>-v</code>	Sets the verbosity level. (Default = NONE)
<code>--strict</code>	Turns on strict tests and display <b>all</b> errors. (Default = False)
<code>--skipCounts</code>	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
<code>outfile</code>	The name of the XML file to create.
<code>infile</code>	The <code>fofn</code> (file-of-file-names) or BAM file(s) to convert into an XML file.

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

`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
<code>infile</code>	The name of the XML file to filter.
<code>outfile</code>	The name of the output filtered XML file.
<code>filters</code>	The values to filter on. ( <b>Example:</b> <code>rq&gt;0.85</code> )

### 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>, <b>not</b> 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 <b>not</b> 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
<code>infile</code>	The name of the XML file to refresh.

Options	Description
<code>--random</code>	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
<code>infile</code>	The name of the Data Set XML file to modify.
<code>metadata</code>	The <code>.metadata.xml</code> file to load, or Data Set to borrow from.

Options	Description
<code>--outfile OUTFILE</code>	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
<code>infile</code>	The name of the XML file to copy.
<code>outdir</code>	The directory to copy to.

Options	Description
<code>--relative</code>	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
<code>infile</code>	The name of the XML file whose paths should be absolute.

Options	Description
<code>--outdir OUTDIR</code>	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:

```
pballign movie1.subreadset.xml referenceset.xml movie1.alignmentset.xml
pballign 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.

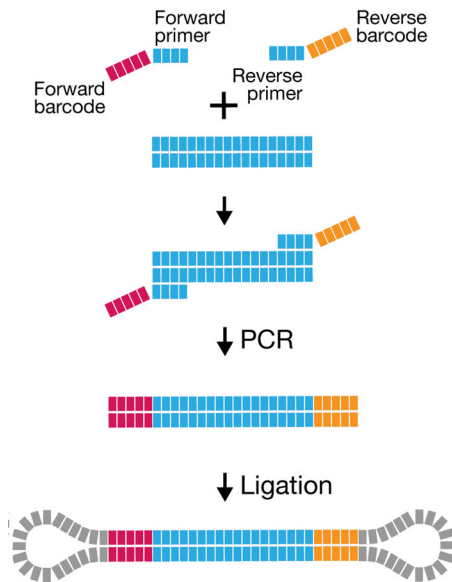
## Demultiplex Barcodes

The **Demultiplex Barcodes** application identifies barcode sequences in PacBio single-molecule sequencing data.

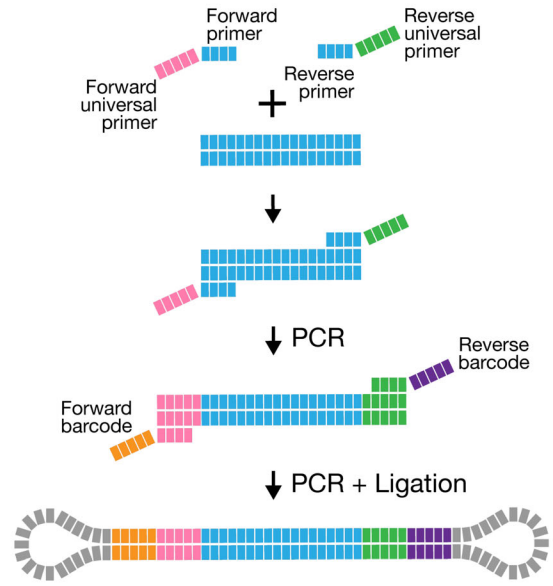
**Demultiplex Barcodes** can demultiplex samples that have a unique per-sample barcode pair and were pooled and sequenced on the same SMRT® Cell. There are four different methods for barcoding samples with PacBio technology:

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

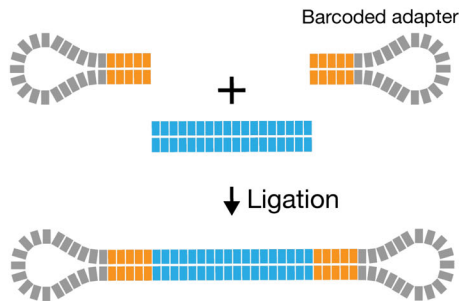
### 1. Sequence-Specific Primers



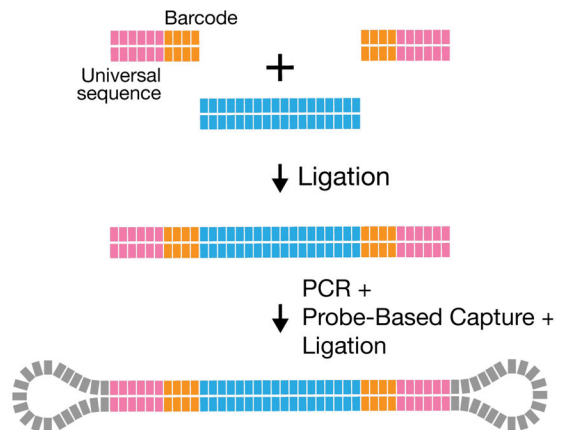
### 2. Barcoded Universal Primers



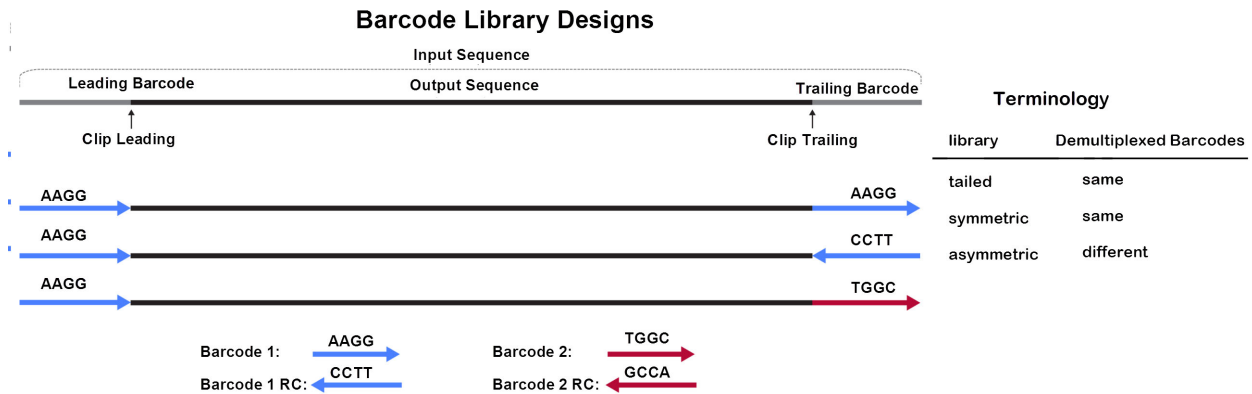
### 3. Barcoded Adapters



### 4. Probe-Based Linear Barcoded Adapters



In addition, there are three different barcode library designs. **Demultiplex Barcodes** supports raw subread and CCS reads demultiplexing.



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 read: --same --ccs
```

## Asymmetric options

Raw: --different

CCS reads: --different --ccs

## Example execution

```
lima m54317_180718_075644.subreadset.xml \  
Sequel_RSII_384_barcode_v1.barcode.v1.xml \  
m54317_180718_075644.demux.subreadset.xml \  
--different --peek-guess
```

Options	Description
--same	Retains only reads with the <b>same</b> barcodes on both ends of the insert sequence, such as symmetric and tailed designs.
--different	Retains only reads with <b>different</b> barcodes on both ends of the insert sequence, asymmetric designs. Enforces <code>--min-passes ≥ 1</code> .
--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 <b>barcode score</b> 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, PacBio 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) <b>Example:</b> 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, <b>all</b> 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
<code>--peek-guess</code>	Sets the following options: <code>--peek 50000 --guess 45 --guess-min-count 10</code> . 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 <code>--ccs</code> then the barcode score threshold is increased by <code>--guess 75</code> .
<code>--single-side</code>	Identifies barcodes in molecules that only have barcodes adjacent to one adapter.
<code>--window-size-mult</code> <code>--window-size-bp</code>	The candidate region size multiplier: <code>barcode_length * multiplier</code> . (Default = 3) Optionally, you can specify the region size in base pairs using <code>--window-size-bp</code> . If set, <code>--window-size-mult</code> is ignored.
<code>--num-threads n</code>	Spawns <code>n</code> threads; 0 means use all available cores. This option also controls the number of threads used for BAM and PBI compression. (Default = 0)
<code>--chunk-size n</code>	Specifies that each thread consumes <code>n</code> ZMWs per chunk for processing. (Default = 10).
<code>--no-bam</code>	Does <b>not</b> produce BAM output. Useful if only reports are of interest, as run time is shorter.
<code>--no-pbi</code>	Does <b>not</b> produce a <code>.bam.pbi</code> index file. The on-the-fly <code>.bam.pbi</code> file generation buffers the output data. If you do not need a <code>.bam.pbi</code> index file for SMRT Link import, use this option to decrease memory usage to a minimum and shorten the run time.
<code>--no-reports</code>	Does <b>not</b> produce any reports. Useful if only demultiplexed BAM files are needed.
<code>--dump-clips</code>	Outputs all clipped barcode regions generated to the <code>&lt;prefix&gt;.lima.clips</code> file.
<code>--dump-removed</code>	Outputs all records that did <b>not</b> pass the specified thresholds, or are without barcodes, to the <code>&lt;prefix&gt;.lima.removed.bam</code> file.
<code>--split-bam</code> <code>--split-bam-named</code>	Specifies that each barcode has its own BAM file called <code>prefix.idxBest-idxCombined.bam</code> , such as <code>prefix.0-0.bam</code> . Optionally, <code>--split-bam-named</code> names the files by their barcode names instead of their barcode indices.
<code>--isoseq</code>	Removes primers as part of the Iso-Seq® pipeline. See “Demultiplexing Iso-Seq® data” on page 33 for details.
<code>--bad-adapter-ratio n</code>	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 II systems and Sequel IIe 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
CATGTCCCTCAGTTAAGTTACAA
>m54007_170702_064558/4850602/6582_6605 bq:37 bc:11
TTTGTACTAAGTATACCAATAG
>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. 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 four 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 cDNA Primer primer set, included with the SMRT Link installation.

Users following the standard Iso-Seq Express protocol **without** multiplexing, or running a Data Set that has **already** been demultiplexed (either using Run Design or the SMRT® Analysis application) should use this default option.

```
>IsoSeq_5p
GCAATGAAGTCGCAGGGTTGGG
>IsoSeq_3p
GTACTCTGCGTTGATACCACTGCTT
```

**Example 2:** The Iso-Seq 12 Barcoded cDNA Primers set, included with the SMRT Link installation.

Users using barcoded cDNA primers listed in the **Appendix 3 - Recommended barcoded NEBNext single cell cDNA PCR primer and Iso-Seq Express cDNA PCR primer sequences** section of the document

---

**Procedure & checklist - Preparing Iso-Seq<sup>®</sup> libraries using SMRTbell Prep Kit 3.0**, should select this option.

```
>bc1001_5p
CACATATCAGAGTGCGGCAATGAAGTCGCAGGGTTGGGG
>bc1002_5p
ACACACAGACTGTGAGGCAATGAAGTCGCAGGGTTGGGG
...
```

(There are a total of 24 sequence records, representing 12 pairs of F/R barcoded cDNA primers.)

**Example 3:** An example of a custom cDNA primer set. 4 tissues were multiplexed using barcodes on the 3' end only.

```
>IsoSeq_5p
GCAATGAAGTCGCAGGGTTGGG
>dT_BC1001_3p
AAGCAGTGGTATCAACGCAGAGTACCACATATCAGAGTGCG
>dT_BC1002_3p
AAGCAGTGGTATCAACGCAGAGTACACACACAGACTGTGAG
>dT_BC1003_3p
AAGCAGTGGTATCAACGCAGAGTACACACATCTCGTGAGAG
>dT_BC1004_3p
AAGCAGTGGTATCAACGCAGAGTACCACGCACACACGCGCG
```

**Example 4:** Special Handling for the TeloPrime cDNA Kit

The Lexogen TeloPrime cDNA kit contains **As** in the 3' primer that **cannot** be differentiated from the polyA tail. For best results, remove the **As** from the 3' end as shown below:

```
>TeloPrimeModified_5p
TGGATTGATATGTAATACGACTCACTATAG
>TeloPrimeModified_3p
CGCCTGAGA
```

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) primers are used in downstream analyses. Good libraries reach >75% CCS reads passing the Demultiplex Barcodes filters.

**BAM tags**

In SMRT Link v11.1, `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_barcode_v3.barcode.v3.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
<code>-o, --output</code>	Name of output ZIP file. (Default = <code>datasets_&lt;timestamp&gt;.zip</code> )
<code>--keep-parent-ref</code>	Keeps the reference to the parent Data Set when archiving a demultiplexed child Data Set.
<code>--no-scrap</code>	Excludes the <code>scrap.bam</code> file if present in the XML file.
<code>-h, --help</code>	Displays help information and exits.
<code>--log-file</code>	Writes the log to a file. (Default = <code>stderr</code> )
<code>--log-level</code>	Specifies the log level; values are <code>[ERROR, DEBUG, INFO, WARN]</code> . (Default = <code>WARN</code> )
<code>--logback</code>	Override all logger configuration using a specified <code>logback.xml</code> file.
<code>--log2stdout</code>	If <code>True</code> , log output is displayed to the console. (Default = <code>False</code> )
<code>--debug</code>	Alias for setting the log level to <code>DEBUG</code> . (Default = <code>False</code> )
<code>--quiet</code>	Alias for setting the log level to <code>ERROR</code> . (Default = <code>False</code> )
<code>--verbose</code>	Alias for setting the log level to <code>INFO</code> . (Default = <code>False</code> )

### 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 <code>logback.xml</code> file.
--log2stdout	If <code>True</code> , log output is displayed to the console. (Default = <code>False</code> )
--debug	Alias for setting the log level to <code>DEBUG</code> . (Default = <code>False</code> )
--quiet	Alias for setting the log level to <code>ERROR</code> . (Default = <code>False</code> )
--verbose	Alias for setting the log level to <code>INFO</code> . (Default = <code>False</code> )

### 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 PacBio's standard BAM format.
- A FASTA file that follows the PacBio 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
<code>-j</code>	Specifies the number of worker processes to use.
<code>--algorithm=</code>	Specifies the variant-calling algorithm to use; values are <code>plurality</code> , <code>arrow</code> and <code>poa</code> . (Default = <code>arrow</code> )
<code>-r</code>	Specifies the FASTA reference file to use.
<code>-o</code>	Specifies the output file format; values are <code>.gff</code> , <code>.vcf</code> , <code>.fasta</code> , and <code>.fastq</code> .
<code>--maskRadius</code>	When using the <code>arrow</code> algorithm, setting this option to a value <code>N</code> greater than 0 causes <code>gcpp</code> 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 <b>may</b> cause worse performance.
<code>--minConfidence MINCONFIDENCE</code> <code>-q MINCONFIDENCE</code>	Specifies the minimum confidence for a variant call to be output to variants.{gff,vcf} (Default = 40)
<code>--minCoverage MINCOVERAGE</code> <code>-x MINCOVERAGE</code>	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 approach 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 (sequence database 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 `pbcrumwell run` command, with the `pb_assembly_hifi` parameter to specify the application. See [“pbcrumwell” on page 80](#) for details.

---

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

```
pbcrumwell 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:

```
pbcrumwell 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:

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

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

```
pbcrumwell 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 `pbcrumwell` 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
<code>-e, --eid_ccs</code>	NONE	Optional parameter, required if <code>--task-option reads &lt;input&gt;</code> is <b>not</b> specified. This is a SMRT Link-specific input parameter and supports <b>only</b> PacBio Consensusreadset XML files as input.
<code>--task-option reads</code>	NONE	Optional parameter, required if <code>-e &lt;input&gt;</code> is <b>not</b> specified. Supports multiple input formats: FASTA, FASTQ, BAM, XML, FOFN and gzipped versions of FASTA/FASTQ.
<code>--task-option ipa2_genome_size</code>	0	The approximate number of base pairs expected in the genome. This is used only for downsampling; if the value is $\leq 0$ , downsampling is disabled. <b>Note:</b> It is better to slightly overestimate rather than underestimate the genome length to ensure good coverage across the genome.
<code>--task-option ipa2_downsampled_coverage</code>	0	The input Data Set can be downsampled to a desired coverage, provided that both the <code>ipa2_downsampled_coverage</code> and <code>ipa2_genome_size</code> options are specified and $>0$ . Downsampling applies to the <b>entire</b> assembly process, including polishing. This parameter selects reads randomly, using a fixed random seed for reproducibility.
<code>--task-option ipa2_advanced_options</code>	NONE	A semicolon-separated list of KEY=VALUE pairs. New line characters are not accepted. (These are described later in this document.)
<code>--task-option ipa2_run_polishing</code>	TRUE	Enables or disables the polishing stage of the workflow. Polishing can be disabled to perform fast draft assemblies.
<code>--task-option ipa2_run_phasing</code>	TRUE	Enables or disables the phasing stage of the workflow. Phasing can be disabled to assemble haploid genomes, or to perform fast draft assemblies.
<code>--task-option ipa2_run_purge_dups</code>	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.
<code>--task-option ipa2_ctg_prefix</code>	<code>.ctg</code>	The prefix used to label the output generated contigs.
<code>--task-option ipa2_reads_db_prefix</code>	<code>reads</code>	The prefix of the sequence and seed databases which will be used internally for assembly.
<code>--task-option ipa2_cleanup_intermediate_files</code>	TRUE	Removes intermediate files from the run directory to save space.
<code>--task-option dataset_filters</code>	NONE	<b>(General pbacromwell option)</b> A semicolon-separated ( <b>not</b> comma-separated) list of other filters to add to the Data Set.
<code>--task-option filter_min_qv</code>	20	<b>(General pbacromwell option)</b> Phred-scale integer QV cutoff for filtering HiFi reads. The default for <b>all</b> applications is 20 (QV 20), or 99% predicted accuracy.
<code>--task-option downsample_factor</code>	0	Downsampling factor applied directly to the input Data Set. This parameter is <b>not</b> related to <code>ipa2_downsampled_coverage</code> .
<code>--task-option mem_scale_factor</code>	8	Controls the amount of requested memory for individual Cromwell tasks in the workflow. The default value of 8 is good for larger genomes, but it may be too much memory for smaller genomes.

Option	Default value	Description
<code>--config</code>	NONE	<b>(General pbcromwell option)</b> Java configuration file for running Cromwell.
<code>--nproc</code>	1	<b>(General pbcromwell option)</b> Number of processors, (except 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 28;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. <b>Note:</b> It is better to slightly overestimate rather than underestimate the genome length to ensure good coverage across the genome.
config_coverage	0	The input Data Set can be downsampled to a desired coverage, provided that both the <code>Downsampled coverage</code> and <code>Genome Length</code> parameters are specified and above 0. Downsampling applies to the <b>entire</b> 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 <code>purge_dups</code> 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 <code>config_ovl_filter_opt</code> value internally, and supersedes the manually specified <code>--max-cov</code> and <code>--max-diff</code> 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). <b>Note:</b> 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	<p>Overlap filter options.</p> <p>--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.</p> <p>--minDepth - Option for the chimera filter. The chimera filter is ignored when a local region of a read has coverage lower than this value.</p> <p>The other parameters are:</p> <p>--min-cov - Minimum allowed coverage at either the 5' or the 3' end of a read. If the coverage is <b>below</b> this value, the read is blacklisted and all of the overlaps it is incident with are ignored. This helps remove potentially chimeric reads.</p> <p>--max-cov - Maximum allowed coverage at either the 5' or the 3' end of a read. If the coverage is <b>above</b> 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 <code>autocompute_max_cov</code> 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).</p> <p>--max-diff - Maximum allowed difference between the coverages at the 5' and 3' ends of any particular read. If the coverage is <b>above</b> this value, the read is blacklisted and all of the overlaps it is incident with are ignored. If the <code>autocompute_max_cov</code> option is used, then the same computed value is supplied to this parameter as well.</p> <p>--bestn - Keep at most this many overlaps on the 5' and the 3' side of any particular read.</p> <p>--min-len - Filter overlaps where either A-read or the B-read are shorter than this value.</p> <p>--idt-stage2 - Filter overlaps with identity below 98%.</p> <p>--high-copy-sample-rate - Controls the downsampling of reads from high copy elements to the expected coverage determined by <code>maxCov*rate</code>, where <code>rate</code> is the value of this parameter. If <code>rate</code> is 0, then these high coverage reads are discarded.</p>
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	<p>Overlapping options for the <code>pancake</code> overlapping tool. The options set by this parameter here are passed directly to <code>pancake</code>. For details on <code>pancake</code> options, use <code>pancake -h</code>.</p> <p>The defaults used here are: <code>--one-hit-per-target</code> which keeps only the best hit in case there are multiple possible overlaps between a pair of reads (tandem repeats); and <code>--min-idt 96</code> which will filter out any overlap with identity lower than 96%.</p>
config_phasing_opt	NONE	Options for the phasing tool <code>nighthawk</code> . The options set by this parameter are passed directly to <code>nighthawk</code> . For details on <code>nighthawk</code> options, use <code>nighthawk -h</code> .

Advanced parameters	Default value	Description
<code>config_phasing_split_opt</code>	<code>--split-type noverlaps --limit 3000000</code>	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: <code>--split-type noverlaps</code> which splits the chunks by the number of overlaps; and <code>--limit 3000000</code> 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.
<code>config_seeddb_opt</code>	<code>-k 28 -w 120 --space 1</code>	Options to control the seed computation. These options are passed directly to the <code>pancake seeddb</code> command.  Defaults: <code>-k 28</code> is the k-mer size of 28 bp; <code>-w 120</code> is the minimizer window size of 120 bp; and <code>--space 1</code> 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 <code>pancake seeddb -h</code> .
<code>config_seqdb_opt</code>	<code>--compression 1</code>	Options to control the construction of the sequence database. These options are passed directly to the <code>pancake seqdb</code> command.  Current default is <code>--compression 1</code> which turns on the 2-bit encoding compression of the sequences.  For more details on these and other options, use <code>pancake seqdb -h</code> .
<code>config_use_hpc</code>	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.
<code>config_use_seq_ids</code>	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.
<code>config_purge_map_opt</code>	<code>--min-map-len 1000 --min-idt 98.0 --bestn 5</code>	This option is used to control the mapping of the reads to contigs for the <code>purge_dups</code> tool. The mapper used is <code>pancake</code> , and the options set by this parameter are used directly by <code>pancake</code> . For details on <code>pancake</code> options, use <code>pancake -h</code> .  Option <code>--min-map-len 1000</code> removes any alignment which did not span more than 1000 bp during the mapping process; <code>--min-idt 98.0</code> removes any alignment with identity below 98.0%, and <code>--bestn 5</code> 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
<code>config_purge_dups_calcuts</code>	NONE	<p>The third-party tool <code>purge_dups</code> can accept user-defined cutoffs for purging. On some genomes, the automated computation of the cutoffs in <code>purge_dups</code> can result in suboptimal values, and in this case a user can specify them manually.</p> <p>This option is passed directly to the <code>purge_dups_calcuts</code> tool. For details on the possible values that can be passed to this tool, use <code>ipa_purge_dups_calcuts</code> without parameters.</p> <p>Relevant parameters include:</p> <ul style="list-style-type: none"> <li>-l INT Lower bound for read depth.</li> <li>-m INT Transition between haploid and diploid.</li> <li>-u INT Upper bound for read depth.</li> </ul>
<code>config_m4filt_high_copy_sample_rate</code>	1.0	<p>This option is passed to the <code>--high-copy-sample-rate</code> parameter of the overlap filter, which controls the downsampling of reads from high copy elements to the expected coverage determined by <code>maxCov*rate</code>, where <code>rate</code> is the value of this parameter. If 0, then these high coverage reads are discarded.</p> <p><b>Note:</b> This parameter supersedes the <code>config_ovl_filter_opt</code> options.</p>
<code>config_max_polish_block_mb</code>	100	<p>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).</p>
<code>config_layout_opt</code>	NONE	<p>This value is passed directly to the assembly layout stage. To get a list of valid options for this parameter, enter <code>ipa2_ovlp_to_graph -h</code> on the command line.</p>

---

## 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 24 for details. If HiFi reads have **not** been generated on the instrument, run CCS analysis first. See “[ccs](#)” on page 8 for details.
  - Provide the proper barcode sequences:  
Barcoded M13 Primer Plate.
  - 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 51 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 `pbserve`:  
`--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 `Ns` for each sample.
- `pb_sars_cov2_kit.frag_fasta_zip`: Zipped file of consensus sequences, split on `Ns`, 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
<code>--task-option sample_wells_csv</code>	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 <b>must</b> 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)
<code>--task-option min_coverage</code>	Specifies the minimum read coverage. <b>Below</b> this value, the consensus sequence will be set to Ns and no variants are called. (Default = 4)
<code>--task-option min_alt_freq</code>	Specifies that only variants whose frequency is <b>greater</b> 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)
<code>--task-option min_bq</code>	Specifies that reads with barcode scores <b>below</b> this minimum value are <b>not</b> included in analysis. (Default = 80)
<code>--task-option mimux_overrides</code>	Specifies additional options to pass to the <code>mimux</code> preprocessing tool for trimming and filtering reads by probe sequences. Options should be entered in space-separated format. Available options include: <code>--max-len</code> : Specifies the maximum sequence length. (Default = 800) <code>--same</code> : Specifies that only reads with arms sequences from the same probe are used.
<code>--task-option probes_fasta</code>	Specifies a FASTA file containing probes sequences if using probes <b>other</b> than those supplied with the HiFiViral SARS-CoV-2 kit.

## 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 PacBio 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 99<sup>th</sup> 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
<code>--gff FILENAME</code>	GFF format.
<code>--csv FILENAME</code>	Comma-separated value format.
<code>--bigwig FILENAME</code>	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.
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 bulk and single-cell sequencing data generated on PacBio instruments. The analysis can be performed *de novo*, without a reference genome. If a reference genome is available, an optional collapse step to produce unique isoform files based on genomic coordinates is available. The input to `isoseq3` tools should be HiFi (CCS) reads in BAM format.

### Recommended commands for bulk Iso-Seq® Analysis

Command	Description	Output format	Recommended next step
<code>lima</code>	Remove cDNA primers.	<code>fl.bam</code>	<code>isoseq3 refine</code>
<code>isoseq3 refine</code>	Remove polyA tail and artificial concatemers.	<code>flnc.bam</code>	<code>isoseq3 cluster</code>
<code>isoseq3 cluster</code>	<i>De novo</i> isoform-level clustering.	<code>unpolished.bam</code>	<code>pbmm2</code>
<code>pbmm2</code>	Align to the genome.	<code>aligned.sorted.bam</code>	<code>isoseq3 collapse</code>
<code>isoseq3 collapse</code>	Collapse redundant transcripts based on exonic structures.	<code>collapsed.gff</code>	<code>pigeon</code>

### Recommended commands for single-cell Iso-Seq® Analysis

Command	Description	Output format	Recommended next step
<code>lima</code>	Remove cDNA primers.	<code>fl.bam</code>	<code>isoseq3 tag</code>
<code>isoseq3 tag</code>	Extract UMIs and cell barcodes.	<code>fl.tagged.bam</code>	<code>isoseq3 refine</code>
<code>isoseq3 refine</code>	Remove polyA tail and artificial concatemers.	<code>flnc.bam</code>	<code>isoseq3 correct</code>
<code>isoseq3 correct</code>	Correct cell barcodes and tag reads that are real cells.	<code>flnc.corrected.bam</code>	<code>isoseq3 bcstats</code>
<code>isoseq3 bcstats</code>	Summarize barcode statistics for real/non-real cells.	<code>bcstats.reports.tsv</code>	<code>isoseq3 dedup</code> or <code>isoseq3 groupdedup</code>
<code>isoseq3 dedup</code> or <code>isoseq3 groupdedup</code>	Deduplicate UMIs.	<code>dedup.bam</code>	<code>pbmm2</code>
<code>pbmm2</code>	Align to the genome.	<code>aligned.sorted.bam</code>	<code>isoseq3 collapse</code>
<code>isoseq3 collapse</code>	Collapse redundant transcripts based on exonic structures.	<code>collapsed.gff</code>	<code>pigeon</code>

### Usage

```
isoseq3 <tool>
```

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits

---

## Typical bulk Iso-Seq workflow

1. 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 24](#) for details on the `lima` tool.

2. Identify and remove polyA tails; also remove artificial concatemers. The output are full-length, non-concatemer (FLNC) reads:

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

3. Cluster FLNC reads at the isoform levels to generate consensus transcript isoform sequences. This generates `unpolished.hq.bam` and `unpolished.hq.fasta.gz` files, which are the high-quality (HQ) transcripts that should be analyzed further.

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

4. (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 --do-not-collapse-extra-5exons aligned.sorted.bam out.gff or
isoseq3 collapse --do-not-collapse-extra-5exons aligned.sorted.bam movie.ccs.bam
out.gff
```

See [“pbmm2” on page 89](#) for details.

`refine` command: 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
<code>ccs.demux.bam xml</code>	Input demultiplexed CCS reads BAM or ConsensusReadSet XML file. This is usually the output from running <code>lima</code> with the <code>--isoseq</code> option, such as <code>demux.5p--3p.bam</code> .
<code>primer.fasta xml</code>	Input primer FASTA or BarcodeSet XML file.
<code>flnc.bam xml</code>	Output FLNC BAM or ConsensusReadSet XML file.

Preprocessing	Description
<code>--min-polya-length</code>	Specifies the minimum poly(A) tail length. (Default = 20)
<code>--require-polya</code>	Requires reads to have a poly(A) tail and remove it.
<code>--min-rq</code>	Specifies the minimum CCS read quality. (Default = -1, deactivated)

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

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

### Usage

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

### Example

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

### 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	<code>flnc.bam</code> file or <code>movie.consensusreadset.xml</code> file.
output	<code>unpolished.bam</code> file prefix (the actual output files will be <code>unpolished.hq.bam</code> and <code>unpolished.lq-bam</code> ) or <code>unpolished.transcriptset.xml</code> file.

Options	Description
<code>--s1</code>	Specifies the number of seeds for minimizer-only clustering. (Default = 1000)
<code>--s2</code>	Specifies the number of seeds for DP clustering. (Default = 1000)

Options	Description
<code>--poa-cov</code>	Specifies the maximum number of CCS reads used for POA consensus. (Default = 10)
<code>--use-qvs</code>	Use CCS analysis Quality Values; sets <code>--poa-cov</code> to 100.
<code>--split-bam</code>	Splits BAM output files into a maximum of <code>N</code> files; 0 means no splitting. (Default = 0)
<code>--min-subreads-split</code>	Subread threshold for High-Quality/Low-Quality split; only works with <code>--use-qvs</code> . (Default = 7)
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, ERROR, CRITICAL]. (Default = WARN)
<code>-v, --verbose</code>	Uses verbose output.
<code>-j, --num-threads</code>	Specifies the number of threads to use; 0 means autodetection. (Default = 0)
<code>--log-file</code>	Writes the log to a file. (Default = stdout)

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

### Usage

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

### Example

```
isoseq3 summarize unpolished.bam summary.csv
```

Inputs/outputs	Description
input	unpolished.bam file. (The output from <code>isoseq3 cluster</code> .)
output	summary.csv file.

Options	Description
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, ERROR, CRITICAL]. (Default = WARN)
<code>-v, --verbose</code>	Uses verbose output.
<code>--log-file</code>	Writes the log to file. (Default = stdout)

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

### Usage

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

### Example - Bulk Iso-Seq

```
isoseq3 collapse --do-not-collapse-extra-5exons aligned.sorted.bam out.gff or
isoseq3 collapse --do-not-collapse-extra-5exons aligned.sorted.bam ccs.bam out.gff
```

## Example - Single-Cell Iso-Seq

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

Inputs/outputs	Description
alignments	Alignments mapping transcripts to the reference genome. (BAM or XML file).
ccs.bam	Optional input BAM file containing CCS reads.
out.gff	Collapsed transcripts in GFF 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)
--max-5p-diff	Specifies the maximum allowed 5' difference if on same exon. (Default = 1000)
--max-3p-diff	Specifies the maximum allowed 3' difference if on same exon. (Default = 100)
--do-not-collapse-extra-5exons	Do not collapse 5' shorter transcripts which miss one or multiple 5' exons to a longer transcript.
--max-batch-mem	Specifies the maximum memory for batch loading, in megabytes (MB). Batches can be slightly larger than this value. Value $\leq 0$ loads all data in memory at once. (Default = 4096)
--split-group-size	Specifies that groups larger than this will be linearly split for parallel processing. (Default = 100)
--keep-non-real-cells	Do not skip reads with non-real cells.
--version	Displays program version number and exits.
--log-file	Writes the log to file. (Default = stderr)
--log-level	Specifies the log level; values are [DEBUG, INFO, WARN, ERROR, CRITICAL]. (Default = WARN)
-j, --num-threads	Specifies the number of threads to use; 0 means autodetection. (Default = 0)

**correct** command: Correct group barcodes ( $x_c:z$ ) given a set of known correct barcodes. Additionally, real (as opposed to background RNA) cells are annotated with the `rc` tag after this step. In subsequent steps, such as `isoseq3 groupdedup`, only reads from real cells as indicated by the `rc` tag will be used.

### Usage

```
isoseq3 correct [options] <flnc.bam> <flnc.corrected.bam>
```

Inputs/outputs	Description
flnc.bam	Input BAM file containing full-length non-concatemer reads, tagged with UMIs and cell barcodes.
flnc.corrected.bam	Output BAM file containing barcode-corrected full-length non-concatemer reads.

Options	Description
--barcodes, -B	Specifies a plain text file containing known "true" barcodes, one per line. This include list specifies the barcode-set to which raw cell barcodes are remapped by minimum edit distance. May be gzip-compressed.
--max-edit-distance, -M	Specifies the maximum edit distance for mapping barcodes to those specified by the <code>-barcodes</code> option. Increasing this value increases yield, but potentially introduce errors. (Default = 2)
--filter, -F	Specifies the filtering mode. <ul style="list-style-type: none"> <li><code>missing</code> removes reads which could not be corrected.</li> <li><code>failing</code> removes reads which could not be corrected as well as reads failing the <code>-max-edit-distance</code> threshold.</li> </ul> (Default = none)
--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 when processing; 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)

`tag` command: Remove cell barcodes and unique molecular identifiers (UMIs) from full-length reads and generate tagged full-length transcripts (FL to FLT).

### Usage

```
isoseq3 tag [options] <fl.bam> <fl.tagged.bam>
```

Inputs/outputs	Description
fl.bam	Input BAM file containing full-length reads.
fl.tagged.bam	Output BAM file containing full-length reads, with UMI and cell barcodes extracted and tagged.

Options	Description
--design	Specifies which bases to use as cell/molecular barcodes as part of the barcoding design. (Default = T-8U-10B)
--min-read-length	Specifies the minimum read length after trimming. (Default = 50)
--help, -h	Displays help information and exits.
--version	Displays program version number and exits.



Options	Description
<code>--verbose, -v</code>	Sets the verbosity level.
<code>-j, --num-threads</code>	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
<code>--log-file</code>	Writes the log to a file. (Default = <code>stderr</code> )
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, TRACE, FATAL]. (Default = WARN)

`groupdedup` command: Deduplicate reads grouped by UMIs (barcode-sorted FLTNC to DEDUP). Note that after barcode correction, the BAM file **must** be sorted by the corrected barcode BAM tag (CB) first.

### Usage

```
samtools sort -t CB corrected.bam -o corrected.sorted.bam
isoseq3 groupdedup [options] <fltnc.bam|xml (one or more).>
```

Inputs/outputs	Description
<code>fltnc.bam xml (one or more)</code>	Input BAM file(s) containing full-length non-concatemer transcripts, sorted by corrected barcode BAM tag (CB).
<code>dedup.bam</code>	Output BAM file(s) containing deduplicated reads.

Options	Description
<code>--poa-cov</code>	Specifies the maximum number of CCS reads used to determine <code>poa</code> consensus, using the <code>poa</code> variant-calling algorithm. (Default = 10)
<code>--max-tag-mismatches</code>	Specifies the maximum number of mismatches between tags. (Default = 1)
<code>--max-tag-shift</code>	Specifies that tags may be shifted by at maximum of N bases. (Default = 1)
<code>--max-length-difference</code>	Specifies the maximum insert lengths difference. (Default = 50)
<code>--max-insert-pad</code>	Specifies the maximum number of missing flanking bases on either insert side. (Default = 5)
<code>--min-concordance-perc</code>	Specifies the minimum insert alignment concordance, in %. (Default = 97)
<code>--max-insert-gaps</code>	Specifies the maximum number of insert gaps per 20 bp window. (Default = 5)
<code>--barcode-tag, -C</code>	Specifies the BAM cell/group tag by which to group. If not specified, checks the CB and XC tags.
<code>--no-poa</code>	Select the highest-pass read in the data set instead of generating a consensus sequence using the <code>poa</code> variant-calling algorithm.
<code>--keep-non-real-cells</code>	Specifies <b>not</b> to skip reads with non-real cells, as indicated by the <code>rc</code> tag.
<code>--batch-size</code>	Specifies the number of BAM records to load per batch. (Default = 500000)
<code>--help, -h</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.

Options	Description
<code>--verbose, -v</code>	Sets the verbosity level.
<code>-j, --num-threads</code>	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
<code>--log-file</code>	Writes the log to a file. (Default = <code>stderr</code> )
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, TRACE, FATAL]. (Default = WARN)

`bcstats` command: Generates statistics for group barcodes (`CB:Z`, `XC:Z`, or `CR:Z`) and (optionally) molecular barcodes (`XM:Z`, `UR:Z`, or `UB:Z`). This tool should be run after `isoseq3 correct` to get the `rc` tag, which indicates which reads come from real cells (as opposed to ambient RNA).

### Usage

```
isoseq3 bcstats [options] <Input BAMs>
```

Inputs	Description
<code>input.bam</code> (one or more)	Input BAM file(s) containing full-length non-concatemer transcripts.

Options	Description
<code>--molecular, -U</code>	Specifies statistics for molecular barcodes (UMIs) as well as cell barcodes; this produces a <code>.tsv</code> file with one line for each cell barcode and one line for each molecular barcode.
<code>-o, --output</code>	Specifies an output <code>.tsv</code> file of statistics for input BAM files. (Default = <code>/dev/stdout</code> )
<code>-R, --json</code>	Specifies the path to an output JSON report. (Default = <code>/dev/stderr</code> )
<code>--deduplicated, -D</code>	Specifies that the output is deduplicated. This allows for faster analysis and sanity checks across versions.
<code>--batch-size, -Z</code>	Specifies the batch size for processing. (Default = 65536)
<code>--percentile, -P</code>	Specifies the percentile to use when calculating real vs non-real cells. This option is relevant <b>only</b> when <code>RealCellCalculationMethod</code> ( <code>--method</code> ) is set to <code>percentile</code> . (Default = 99)
<code>-T, --target</code>	Specifies whether to determine real vs non-real cells by read count ( <code>readcount</code> ) or UMI count ( <code>umicount</code> ). (Default = <code>umicount</code> )
<code>-M, --method</code>	Specifies whether to determine real vs non-real cells using the Knee-finding ( <code>knee</code> ) or the percentile-based method ( <code>percentile</code> ). (Default = <code>knee</code> )
<code>--help, -h</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--verbose, -v</code>	Sets the verbosity level.
<code>-j, --num-threads</code>	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
<code>--log-file</code>	Writes the log to a file. (Default = <code>stderr</code> )
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, TRACE, FATAL]. (Default = WARN)

`dedup` command: Deduplicate reads grouped by UMIs. **Note:** This tool has been superseded by the `groupdedup` tool.

## Usage

```
isoseq3 dedup [options] <fltnc.bam|xml> <dedup.bam|xml>
```

Inputs/outputs	Description
fltnc.bam xml (one or more)	Input BAM file(s) containing full-length non-concatemer transcripts, sorted by corrected barcode BAM tag (CB).
dedup.bam xml	Output BAM file(s) or XML file(s) containing deduplicated reads.

Options	Description
<code>--poa-cov</code>	Specifies the maximum number of CCS reads used to determine poa consensus, using the poa variant-calling algorithm. (Default = 10)
<code>--max-tag-mismatches</code>	Specifies the maximum number of mismatches between tags. (Default = 1)
<code>--max-tag-shift</code>	Specifies that tags may be shifted by at maximum of N bases. (Default = 1)
<code>--max-insert-length-diff</code>	Specifies the maximum insert lengths difference. (Default = 50)
<code>--max-insert-pad</code>	Specifies the maximum number of missing flanking bases on either insert side. (Default = 5)
<code>--min-concordance-perc</code>	Specifies the minimum insert alignment concordance, in %. (Default = 97)
<code>--max-insert-gaps</code>	Specifies the maximum number of insert gaps per 20 bp window. (Default = 5)
<code>--keep-non-real-cells</code>	Specifies <b>not</b> to skip reads with non-real cells, as indicated by the rc tag.
<code>--help, -h</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--verbose, -v</code>	Sets the verbosity level.
<code>-j, --num-threads</code>	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
<code>--log-file</code>	Writes the log to a file. (Default = stderr)
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARN, TRACE, FATAL]. (Default = WARN)

---

**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  $\geq 0.99$ , 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
<code>input_file.bam</code>	Input aligned BAM file containing CCS reads, which must be PacBio-compliant, that is, <code>cigar M</code> is forbidden.
<code>output_file.html</code>	Output report HTML file.

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

Restrictions	Description
<code>--region, -r</code>	Specifies the genomic region of interest; reads are clipped to that region. Empty means <b>all</b> reads.
<code>--drm-only, -k</code>	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.
<code>--min-perc, -m</code>	Specifies the minimum variant percentage to report. <b>Example:</b> <code>--min-perc 1</code> will only show variant calls with an observed abundance of more than 1%. (Default = 0)
<code>--max-perc, -n</code>	Specifies the maximum variant percentage to report. <b>Example:</b> <code>--max-perc 95</code> will only show variant calls with an observed abundance of less than 95%. (Default = 100)

Chemistry override (specify both)	Description
<code>--sub, -s</code>	Specifies the substitution rate. Use to override the learned rate. (Default = 0)
<code>--del, -d</code>	Specifies the deletion rate. Use to override the learned rate. (Default = 0)

Options	Description
<code>--help, -h</code>	Displays help information and exits.
<code>--verbose, -v</code>	Sets the verbosity level.
<code>--version</code>	Displays program version number and exits.
<code>--debug</code>	Returns all amino acids, irrespective of their relevance.
<code>--mode-phasing, -p</code>	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 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

HIV HXB2			Reverse Transcriptase					Sample Variants	Affected Drugs*
Codon	AA	Pos	AA	Codon	%	Coverage			
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	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*
A A C	N	47	S	A G T	0.95	2924	
Protease							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
C G A	R	8	X	T G A	0.98	2931	
Reverse Transcriptase							
HIV HXB2			Sample Variants				
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*
A T G	M	41	L	T T G	0.99	2903	ABC + DDI + TDF + D4T + ZDV
A A A	K	65	R	A G A	1	2577	3TC + FTC + ABC + DDI + TDF + D4T
T T A	L	100	F	T T T	0.85	2819	
T A T	Y	181	C	T G T	0.95	2939	NVP + EFV + ETR + RPV
G G A	G	190	A	G C A	1	2941	NVP + EFV + ETR + RPV
A C C	T	215	Y	T A C	0.88	2940	ABC + DDI + TDF + D4T + ZDV

- **Note:** For the predefined configuration `HIV`, 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	<b>Only</b> 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								A	B	C	D	E	F	G	H	I
HXB2		Sample Variants						Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
C G A	R	8	X	T G A	0.98	2931	MGI									
Reverse Transcriptase								A	B	C	D	E	F	G	H	I
HXB2		Sample Variants						Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
A T G	M	41	L	T T G	0.99	2903	ABC + DDI + TDF + D4T + ZDV									
A A A	K	65	R	A G A	1	2577	3TC + FTC + ABC + DDI + TDF + D4T									
G G G	G	99	G	G G T	0.72	2907										
T T A	L	100	F	T T T	0.85	2819	MGI									
T A T	Y	181	C	T G T	0.95	2939	NVP + EFV + ETR + RPV									
G G A	G	190	A	G C A	1	2941	MGI + NVP + EFV + ETR + RPV									
A C C	T	215	Y	T A C	0.88	2940	ABC + DDI + TDF + D4T + ZDV									
Integrase								A	B	C	D	E	F	G	H	I
HXB2		Sample Variants						Haplotypes %								
Codon	AA	Pos	AA	Codon	%	Coverage	Affected Drugs*	92.5	1.2	1.2	1	1	0.8	0.8	0.8	0.7
A A A	K	188	K	A A G	0.92	2923	MGI									

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

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

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

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

**Haplotypes %**

i 2.8 2.2 1.3 1 1 1 1 0.9 0.7 0

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

A	B	C	H
3.2	1.2	1.2	27

---

## Microbial Genome Analysis

The Microbial Genome Analysis application is powered by the IPA HiFi genome assembler and includes a base modification detection feature performed after the assembly.

### Workflow of the Microbial Genome Analysis 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 Data Set to the assembled contigs.
8. Polish assembled contigs using `Racon`.
9. Perform base modification detection.

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 of assembly 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 Genome Analysis application is run using the `pbchromwell` run command, with the `pb_microbial_analysis` parameter to specify the application. See [“pbchromwell” on page 80](#) for details.

To view information on the available Microbial Genome Analysis options, enter:

```
pbchromwell show-workflow-details pb_microbial_analysis
```

The minimum command needed to run the workflow requires the input Data Set.

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

```
pbchromwell run pb_microbial_analysis -e <input.consensusreadset.xml>
```

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

```

pbccromwell run pb_microbial_analysis \
-e <input.consensusreadset.xml> \
--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 Genome Analysis parameters

Option	Default value	Description
-e, --eid_ccs	NONE	This is a SMRT Link-specific input parameter and supports <b>only</b> PacBio Consensusreadset XML files as input.
--task-option reads	NONE	Optional parameter, required if -e <input> is <b>not</b> specified. Supports multiple input formats: FASTA, FASTQ, BAM, XML, FOFN and gzipped versions of FASTA/FASTQ.
--task-option ipa2_genome_size	10M	The approximate number of base-pairs expected in the chromosomal genome. Used only for downsampling in the assembly stages (that is, not in polishing). If value <=0, then downsampling is off. <b>Default:</b> 10M (10 Mega basepairs). <b>Note:</b> ipa2_genome_size is currently the <b>only</b> 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 with respect to the estimated genome_size. (The default genome_size was chosen to be larger than most realistic microbes.) If value <=0, then downsampling is off. <b>Default:</b> 100 <b>Example:</b> 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 downsampling to 200x coverage, simply because of the over-estimate. The cost of extra coverage is greater runtime. The defaults are usually fine.

Option	Default value	Description
--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_length_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 microasm_run_secondary_polish	TRUE	Specifies that an additional round of polishing will be applied after the two stages of assembly have completed.
--task-option run_basemods	TRUE	Specifies that base modification analysis be performed.
--task-option kineticstools_identify_mods	m4C,m6A	Specify the base modifications to identify, in a comma-separated list.
--task-option kineticstools_p_value	0.001	Specifies the probability value cutoff for detecting base modifications.
--task-option motif_min_score	35	Specifies the minimum QMod score used to identify a motif.
--task-option motif_min_fraction	0.30	Specifies the minimum methylated fraction to identify a motif.
--task-option run_find_motifs	TRUE	Specifies that motif-finding be performed.
--task-option dataset_filters	NONE	<b>(General pbcrmwel option)</b> A semicolon-separated ( <b>not</b> comma-separated) list of other filters to add to the Data Set.
--task-option filter_min_qv	20	<b>(General pbcrmwel option)</b> Phred-scale integer QV cutoff for filtering HiFi reads. The default for <b>all</b> applications is 20 (QV 20), or 99% predicted accuracy.

Option	Default value	Description
<code>--task-option log_level</code>	INFO	Specifies the logging (verbosity) level for tools which support this feature. Values are: [TRACE, DEBUG, INFO, WARN, FATAL]
<code>--task-option nproc</code>	1	Specified the number of CPU threads to use.
<code>--task-option max_nchunks</code>	40	Specifies the maximum number of chunks per task.
<code>--task-option tmp_dir</code>	/tmp	Specifies an optional temporary directory for used by several subtools, such as <code>sort</code> .

### Input files

- \*.bam file containing PacBio data.
- \*.xml file 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.
- `pb_microbial_analysis.motifs_csv`: File containing motifs and modifications.

### Advanced assembly options

**Note:** Advanced parameters should be rarely modified. Advanced parameters for the Microbial Genome Assembly workflow are the **same** as those for the Genome Assembly workflow. See [“Advanced parameters” on page 45](#) 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
28;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
<code>-h, --help</code>	Displays help information and exits.
* <code>-f, --fasta</code>	Reference FASTA file.
* <code>-g, --gff</code>	Modifications.gff or .gff.gz file.
<code>-m, --minScore</code>	Specifies the minimum Qmod score to use in motif finding. (Default = 40.0)
* <code>-o, --output</code>	Outputs motifs.csv file.
<code>-x, --xml</code>	Outputs motifs XML file.

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

```
reprocess [options]
```

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

## Output files

Using the `find` command:

- **Output CSV file:** Contains motif information.

Using the `reprocess` command:

- **Output GFF file:** Contains added motif information based on new Modification QV thresholds. The format of the output file is the same as the input file.

**pbacromwell** The `pbacromwell` tool is PacBio’s wrapper for the `cromwell` scientific workflow engine used to power SMRT Link. `pbacromwell` includes advanced utilities for interacting directly with a Cromwell server.

`pbacromwell` 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 `pbacromwell` does **not** interact with SMRT Link services; to run a Cromwell workflow as a SMRT Link job, use `pbservice`. (For details, see “[pbservice](#)” on page 96.)

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

### Usage

```
pbacromwell 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
<code>--output-dir OUTPUT_DIR</code>	Specifies the output directory for <code>cromwell</code> output. (Default = <code>cromwell_out</code> )
<code>--overwrite</code>	Overwrites the output directory, if it exists. (Default = <code>False</code> )
<code>-i INPUTS, --inputs INPUTS</code>	Specifies <code>cromwell</code> inputs and settings as JSON files. (Default = <code>None</code> )
<code>-e ENTRY_POINTS, --entry ENTRY_POINTS</code>	Specifies the entry point Data Set; may be repeated for workflows that take more than one input Data Set. Note that <b>all</b> PacBio workflows require at least <b>one</b> such entry point.
<code>-n NPROC, --nproc NPROC</code>	Specifies the number of processors per task. (Default = <code>1</code> )
<code>-c MAX_NCHUNKS, --max-nchunks MAX_NCHUNKS</code>	Specifies the maximum number of chunks per task. (Default = <code>None</code> )
<code>--target-size TARGET_SIZE</code>	Specifies the target chunk size. (Default = <code>None</code> )
<code>--queue QUEUE</code>	Specifies the cluster queue to use. (Default = <code>None</code> )
<code>-o OPTIONS, --options OPTIONS</code>	Specifies additional <code>cromwell</code> engine options, as a JSON file. (Default = <code>None</code> )
<code>-t TASK_OPTIONS, --task-option TASK_OPTIONS</code>	Specifies workflow- or task-level option as <code>key=value</code> strings, specific to the application. May be specified multiple times for multiple options. (Default = <code>[]</code> )
<code>-b BACKEND, --backend BACKEND</code>	Specifies the backend to use for running tasks. (Default = <code>None</code> )
<code>-r MAX_RETRIES, --maxRetries MAX_RETRIES</code>	Specifies the maximum number of times to retry a failing task. (Default = <code>1</code> )

Options	Description
<code>--tmp-dir TMP_DIR</code>	Specifies an optional temporary directory for <code>cromwell</code> tasks, which <b>must</b> exist on all compute hosts. (Default = None)
<code>--config CONFIG</code>	Specifies a Java configuration file for running <code>cromwell</code> . (Default = None)
<code>--dry-run</code>	Specifies that <code>cromwell</code> is <b>not</b> executed, but instead writes out final inputs and then exits. (Default = True)
<code>workflow</code>	Specifies the workflow ID (such as <code>pb_ccs</code> or <code>cromwell.workflows.pb_ccs</code> for PacBio workflows only) or a path to a Workflow Description Language (WDL) source file.

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

### Examples

Show available PacBio-developed workflows:

```
pbccromwell show-workflows
```

Show details for a PacBio workflow:

```
pbccromwell show-workflow-details pb_ccs
```

Generate `cromwell.conf` with HPC settings:

```
pbccromwell configure --default-backend SGE --output-file cromwell.conf
```

Launch a PacBio workflow:

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

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

`pbccromwell 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 Data Sets 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, see the official documentation [here](#).

### Workflow examples

Run the CCS analysis:

```
pbccromwell 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:

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

Run a user-defined workflow:

```
pbccromwell 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:

```
pbccromwell 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.

```
pbccromwell show-workflow-details pb_ccs  
pbccromwell show-workflow-details cromwell.workflows.pb_ccs
```

`pb cromwell show-workflow-details` command: Display details about a specified PacBio workflow, including input files and task options.

### Usage

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

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

`pb cromwell 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 `pb cromwell run` using the `--config` argument.

### Usage

```
pb cromwell 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
<code>--port PORT</code>	Specifies the port that <code>cromwell</code> should listen to. (Default = 8000)
<code>--local-job-limit LOCAL_JOB_LIMIT</code>	Specifies the maximum number of local jobs/tasks that can be run at once. (Default = 10)
<code>--jms-job-limit JMS_JOB_LIMIT</code>	Specifies the maximum number of jobs/tasks that can be submitted to the queueing system at one time. (Default = 500)
<code>--db-port DB_PORT</code>	Specifies the database port for <code>cromwell</code> to use; if undefined, database configuration is omitted. (Default = None)
<code>--db-user DB_USER</code>	Specifies the user name used to connect to Postgres. (Default = <code>smrtlink_user</code> )
<code>--db-password DB_PASSWORD</code>	Specifies the password used to connect to Postgres.
<code>--default-backend DEFAULT_BACKEND</code>	Specifies the default job execution backend. Choices are: Local, SGE, Slurm, PBS, or LSF. (Default = Local)
<code>--max-workflows MAX_WORKFLOWS</code>	Specifies the maximum number of workflows that <code>cromwell</code> can run at once. (Default = 100)
<code>--output-file OUTPUT_FILE</code>	Specifies the name of the output configuration file. (Default = <code>cromwell.conf</code> )
<code>--timeout TIMEOUT</code>	Specifies the time to wait for task completion before checking the cluster job status. (Default = 600)

---

<b>Options</b>	<b>Description</b>
--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
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	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 Subreads.

`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
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of <code>stderr</code> .



Options	Description																
<code>--log-level</code>	<p>Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL] (Default = WARN)</p> <p><code>--log-level INFO</code> produces a summary report such as:</p> <table border="1"> <thead> <tr> <th>LIBRARY</th> <th>READS</th> <th>UNIQUE MOLECULES</th> <th>DUPLICATE READS</th> </tr> </thead> <tbody> <tr> <td>&lt;Unnamed&gt;</td> <td>25000</td> <td>24948 (99.8%)</td> <td>52 (0.2%)</td> </tr> <tr> <td>SS-lib</td> <td>496</td> <td>493 (99.4%)</td> <td>3 (0.6%)</td> </tr> <tr> <td>TOTAL</td> <td>25496</td> <td>25441 (99.8%)</td> <td>55 (0.2%)</td> </tr> </tbody> </table>	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%)
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%)														
<code>-j, --num-threads</code>	Specifies the number of threads to use, 0 means autodetection. (Default = 0)																

Duplicate marking options	Description
<code>--cross-library, -x</code>	Identifies duplicate reads across sequencing libraries. Libraries are specified in the BAM read group LB tag.

Output options	Description
<code>-rmdup, -r</code>	Excludes duplicates from <code>OUTFILE</code> . (This is redundant when <code>--dup-file</code> is specified.)
<code>--dup-file FILE</code>	Stores duplicate reads in an extra file other than <code>OUTFILE</code> . The format of this file can be different from the output file.
<code>--clobber, -f</code>	Overwrites <code>OUTFILE</code> 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
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	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
<code>--preset</code>	Specifies the alignment mode: <ul style="list-style-type: none"> <li>"SUBREAD" -k 19 -w 10</li> <li>"CCS" -k 19 -w 10 -u</li> <li>"ISOSEQ" -k 15 -w 5 -u</li> <li>"UNROLLED" -k 15 -w 15</li> </ul> The option is <b>not</b> case-sensitive. (Default = SUBREAD)
<code>-k</code>	Specifies the k-mer size, which cannot be larger than 28. (Default = -1)
<code>-w</code>	Specifies the Minimizer window size. (Default = -1)
<code>-u, --no-kmer-compression</code>	Disables homopolymer-compressed k-mer. (Compression is on by default for the SUBREAD and UNROLLED presets.)
<code>--short-sa-cigar</code>	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.fasta|xml|mml> <in.bam|xml|fa|fq> [out.aligned.bam|xml]
```

### Input files

- `*.fasta` file containing reference contigs, or `*.referenceset.xml`, or `*.mml` 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/FASTQ 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/FASTQ 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/FASTQ input.

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>--chunk-size</code>	Processes <code>N</code> records per chunk. (Default = 100)
<code>--sort</code>	Generates a sorted BAM file.
<code>-m, --sort-memory</code>	Specifies the memory per thread for sorting. (Default = 768M)
<code>-j, --alignment-threads</code>	Specifies the number of threads used for alignment. 0 means autodetection. (Default = 0)
<code>-J, --sort-threads</code>	Specifies the number of threads used for sorting. 0 means 25% of <code>-j</code> , with a maximum of 8. (Default = 0)
<code>--sample</code>	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".
<code>--rg</code>	Specifies the read group header line such as <code>'@RG\tID:xyz\tSM:abc'</code> . Only for FASTA/Q inputs.
<code>-y, --min-gap-comp-id-perc</code>	Specifies the minimum gap-compressed sequence identity, in percent. (Default = 70)
<code>-l, --min-length</code>	Specifies the minimum mapped read length, in base pairs. (Default = 50)
<code>-N, --best-n</code>	Specifies the output at maximum <code>N</code> alignments for each read. 0 means no maximum. (Default = 0)
<code>--strip</code>	Removes all kinetic and extra QV tags. The output cannot be polished.
<code>--split-by-sample</code>	Specifies one output BAM file per sample.
<code>--no-bai</code>	Omits BAI index file generation for sorted output.
<code>--unmapped</code>	Specifies that unmapped records be included in the output.
<code>--median-filter</code>	Picks one read per ZMW of median length.
<code>--zmw</code>	Processes ZMW Reads; <code>subreadset.xml</code> input is required. This activates the UNROLLED preset.

Options	Description
<code>--hqregion</code>	Processes the HQ region of each ZMW; <code>subreadset.xml</code> input is required. This activates the UNROLLED preset.

Parameter set options and overrides	Description
<code>--preset</code>	Specifies the alignment mode: <ul style="list-style-type: none"> <li>"SUBREAD" <code>-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</code></li> <li>"CCS" <code>-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</code></li> <li>"ISOSEQ" <code>-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</code></li> <li>"UNROLLED" <code>-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</code></li> </ul> (Default = SUBREAD)
<code>-k</code>	Specifies the k-mer size, which cannot be larger than 28. (Default = -1)
<code>-w</code>	Specifies the Minimizer window size. (Default = -1)
<code>-u, --no-kmer-compression</code>	Disables homopolymer-compressed k-mer. (Compression is on by default for the SUBREAD and UNROLLED presets.)
<code>-A</code>	Specifies the matching score. (Default = -1)
<code>-B</code>	Specifies the mismatch penalty. (Default = -1)
<code>-z</code>	Specifies the Z-drop score. (Default = -1)
<code>-Z</code>	Specifies the Z-drop inversion score. (Default = -1)
<code>-r</code>	Specifies the bandwidth used in chaining and DP-based alignment. (Default = -1)
<code>-o, --gap-open-1</code>	Specifies the gap open penalty 1. (Default = -1)
<code>-O, --gap-open-2</code>	Specifies the gap open penalty 2. (Default = -1)
<code>-e, --gap-extend-1</code>	Specifies the gap extension penalty 1. (Default = -1)
<code>-E, --gap-extend-2</code>	Specifies the gap extension penalty 2. (Default = -1)
<code>-L, --lj-min-ratio</code>	Specifies the long join flank ratio. (Default = -1)
<code>-G</code>	Specifies the maximum intron length; this changes <code>-r</code> . (Default = -1)
<code>-C</code>	Specifies the cost for a non-canonical GT-AG splicing. (Default = -1)
<code>--no-splice-flank</code>	Specifies that you do <b>not</b> 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. See the available parameter sets in the option table shown earlier.

## What other special parameters are used implicitly?

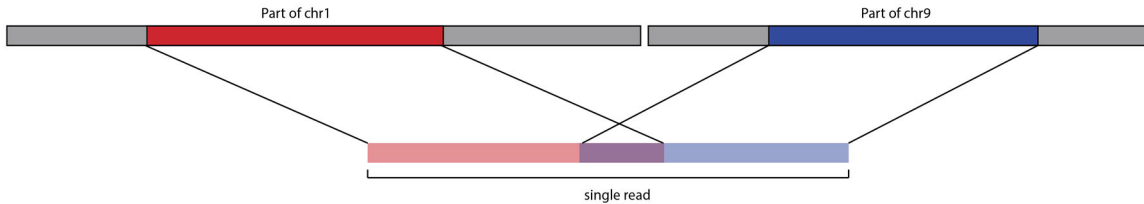
We implicitly use the following `minimap2` parameters:

- 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:** `pbservice` 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
<code>--host=http://localhost</code>	Specifies the server host. Override the default with the environmental variable <code>PB_SERVICE_HOST</code> .
<code>--port=8070</code>	Specifies the server port. Override the default with the environmental variable <code>PB_SERVICE_PORT</code> .
<code>--log-file LOG_FILE</code>	Writes the log to file. (Default = None, writes to stdout.)
<code>--log-level=INFO</code>	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL.] (Default = INFO)
<code>--debug=False</code>	Alias for setting the log level to DEBUG. (Default = False)
<code>--quiet=False</code>	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
<code>--user USERNAME</code>	Specifies the user to authenticate as; this is <b>required</b> if the target host is anything other than <code>localhost</code> .
<code>--ask-pass</code>	Prompts the user to enter a password.
<code>--password PASSWORD</code>	Supplies the password directly. This exposes the password in the shell history (or log files), so this option is <b>not</b> 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.

```
pbserve 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.

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

Required	Description
fasta_path	Path to the FASTA file to import.

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

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

```
pbserve 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:  $\geq 20$  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
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of stdout.
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)
<code>discover</code>	Finds structural variant signatures in read alignments (BAM to SVSIG).
<code>call</code>	Calls structural variants from SV signatures and assign genotypes (SVSIG to VCF).

`pbsv discover` command: Finds structural variant (SV) signatures in read alignments. The input read alignments must be sorted by chromosome position. Alignments are typically generated with `pbbmm2`. The output SVSIG file contains SV signatures.

### Usage

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

Required	Description
<code>ref.in.bam xml</code>	Coordinate-sorted aligned reads in which to identify SV signatures.
<code>ref.out.svsig.gz</code>	Structural variant signatures output.

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>-s, --sample</code>	Overrides sample name tag from BAM read group.
<code>-q, --min-mapq</code>	Ignores alignments with mapping quality $< N$ . (Default = 20)
<code>-m, --min-ref-span</code>	Ignores alignments with reference length $< N$ bp. (Default = 100)
<code>-w, --downsample-window-length</code>	Specifies a window in which to limit coverage, in base pairs. (Default = 10K)
<code>-a, --downsample-max-alignments</code>	Considers up to $N$ alignments in a window; 0 means disabled. (Default = 100)

Options	Description
-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` 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 <code>pbsv discover</code> , 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)



Options	Description
-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 TTGGTGTGGTTGTTTGTGGTTT . 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 PacBio's 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,ConsensusAlignmen
  tSet,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)
--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, ConsensusAlignmen tSet, SubreadSet, BarcodeSet, ContigSet, ReferenceSet, HdfSubreadSet] (Default = None)

Options	Description
<code>--index</code>	Requires index files: <code>.fai</code> or <code>.pbi</code> . (Default = False)
<code>--strict</code>	Turns on additional validation, primarily for Data Set XML. (Default = False)

BAM options	Description
<code>--unaligned</code>	Specifies that the file should contain <b>only</b> unmapped alignments. (Default = None, no requirement.)
<code>--unmapped</code>	Alias for <code>--unaligned</code> . (Default = None)
<code>--aligned</code>	Specifies that the file should contain <b>only</b> mapped alignments. (Default = None, no requirement.)
<code>--mapped</code>	Alias for <code>--aligned</code> . (Default = None)
<code>--contents</code>	Enforces the read type: [SUBREAD, CCS] (Default = None)
<code>--reference REFERENCE</code>	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
```

**pigeon** The `pigeon` tool is a PacBio transcript toolkit that contains tools to classify and filter full-length transcript isoforms into categories (see table below) against a reference annotation.

`pigeon` is based on the [SQANTI3](#) software, and `pigeon` output is compatible with downstream analysis with the [Seurat](#) software.

`pigeon` characterizes isoforms obtained by full-length transcript sequencing. `pigeon` combines the long read-defined transcripts with the reference annotation as well as with other orthogonal data to provide a wide range of descriptors of transcript quality. `pigeon` then generates a comprehensive report to facilitate quality control and filtering of the isoform models.

### Pigeon isoform categories

Category	Description
FSM (Full Splice Match)	The reference and query isoform have the <b>same</b> number of exons and each internal junction matches the positions of the reference. The exact 5' start and 3' end can differ within the first/last exons.
ISM (Incomplete Splice Match)	The query isoform has <b>fewer</b> external exons than the reference, but each internal junction matches the positions of the reference. The exact 5' start and 3' end can differ within the first/last exons.
NIC (Novel In Catalog)	The query isoform does <b>not</b> have a FSM or ISM match, but is using a combination of known donor/acceptor sites.
NNC (Novel Not in Catalog)	The query isoform does <b>not</b> have a FSM or ISM match, and has at least <b>one</b> donor or acceptor site that is <b>not</b> annotated.
Antisense	The query isoform does not have overlap with a same-strand reference gene, but is anti-sense to an annotated gene.
Fusion	The query isoform overlaps two or more reference genes.
More junctions	The query isoform overlaps two or more reference genes, however some junctions are shared by multiple genes.
Genic Intron	The query isoform is <b>completely contained</b> within a reference intron.
Genic Genomic	The query isoform overlaps with introns and exons.
Intergenic	The query isoform is in the intergenic region.

### Usage

```
pigeon [options] <index,classify,filter,report,make-seurat>
```

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of <code>stdout</code> .
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)

`index` command: Creates indices for sorted genomic annotation files. (This is **required** for input to the classification stage.) Acceptable annotation files include GTF for annotations and CAGE peak BED files.

### Usage

```
pigeon index [options] <input file>
```

### Examples

```
pigeon index gencode.v25.annotation.gtf
pigeon index refTSS_v3.1_human_coordinate.hg38.bed
```

Required	Description
Input file	Annotation text file. Annotations <b>must</b> be grouped by reference, with the name in the first column, such as BED or GTF.

Options	Description
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of <code>stdout</code> .
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)

`sort` command: Sorts the mapped collapsed GFF file. (This is **required** for input to the classification stage.)

### Usage

```
pigeon sort [options] <Input file>
```

### Example

```
pigeon sort collapsed.gff -o sorted.gf
```

Required	Description
Input file	Mapped collapsed isoform GFF file.

Options	Description
<code>-o</code>	Specifies the output file name. If <b>not</b> specified, the output file name is the input file name with the suffix <code>.sorted</code> .
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of <code>stdout</code> .
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)

`classify` command: Classifies isoforms according to their structural categories and detects novel isoforms, as compared to known genome annotations.

## Usage

```
pigeon classify [options] <isoforms.gff> <annotations.gtf> <reference.fa>
```

Required	Description
isoforms.gff	Sorted collapsed GFF file containing the Isoforms to classify.
annotations.gtf	GTF file containing reference annotations. <b>Note:</b> The associated <code>.pgi</code> file is <b>required</b> . (Use the <code>pigeon index</code> command to generate one.)
reference.fa	FASTA file containing references; this may be gzipped <code>[.gz]</code> . <b>Note:</b> The associated <code>.fai</code> file is <b>required</b> . (Use the <code>samtools faidx</code> command to generate one.)

Input/output options	Description
<code>-r, --ref</code>	Specifies a ReferenceSet dataset XML file. May be used in place of the reference annotation and sequence positional arguments, but the dataset <b>must</b> provide these inputs. May (optionally) provide supplemental reference resources such as the polyA motif and CAGE peak input files.
<code>-d, --out-dir</code>	Specifies the destination directory for all output files. (Default = <code>[.]</code> )
<code>-o, --out-prefix</code>	Specifies a prefix for all non-matrix output files. If <b>not</b> specified, the primary input isoform file is used to determine the prefix. <b>Example:</b> An input file of <code>my_isoforms.gff</code> yields the output file name prefix <code>my_isoform</code> .
<code>--fl, --flnc</code>	Specifies the name of a full-length PacBio abundance file.
<code>--cage-peak</code>	Specifies the name of a CAGE (Cap Analysis of Gene Expression) data file in BED format. <b>Note:</b> The associated <code>.pgi</code> file is <b>required</b> . (Use the <code>pigeon index</code> command to generate one.) See <a href="#">here</a> for more information on CAGE.
<code>--poly-a</code>	Specifies the name of a text file containing a ranked list of polyA motifs, one per line.
<code>-c, --coverage</code>	Specifies junction coverage data. Associated <code>.pgi</code> file is required. Use <code>pigeon index</code> to generate one.
<code>--gene-id</code>	Specifies the use of the gene ID instead of the gene name, such as <code>ENSG00000206195.11</code> rather than <code>DUXAP8</code> .

Algorithm setting	Description
<code>--sites</code>	Specifies a comma-separated list of splice sites to be considered as canonical. (Default = <code>GTAG, GCAG, ATAC</code> )
<code>--window</code>	Specifies the size of the window (in base pairs) in the genomic DNA screened for Adenine content downstream of TTS. (Default = <code>20</code> )
<code>--min-ref-length</code>	Specifies the minimum reference transcript length, in base pairs. (Default = <code>200</code> )
<code>--is-fusion</code>	Specifies that the inputs are fusion isoforms.
<code>--polya-search-distance</code>	Specifies the distance (in base pairs) to look upstream of the 3' end for the highest ranking polyA motif signal. (Default = <code>50</code> )



Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
-j, --num-threads	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
--log-file	Logs to a file, instead of <code>stdout</code> .
--log-level	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)

`filter` command: Removes isoform classifications that indicate amplification artifacts such as intrapriming or template switching.  
**Note:** `pigeon filter` can **only** be called after `pigeon classify` is first called.

### Usage

```
pigeon filter [options] <classification_file>
```

### Examples

```
pigeon filter classification.txt
```

```
pigeon filter classification.txt --isoforms sorted.gff
```

Required	Description
classification file	Classification.txt output file from <code>pigeon filter</code> .

Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
--log-file	Logs to a file, instead of <code>stdout</code> .
--log-level	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL.] (Default = WARN)
--isoforms	Specifies the generation of a filtered GFF. This <b>requires</b> that you specify the GFF file that was used as input to <code>pigeon classify</code> .

`report` command: Reports gene saturation. Gene saturation can be determined by subsampling the classification output and determining the number of unique genes at each subsample size.

### Usage

```
pigeon report [options] <classification.txt> <subsample.txt>
```

### Example

```
pigeon report <classification.filtered_lite_classification.txt> <saturation.txt>
```

Required	Description
classification.txt	Input classification text file. Could be after <code>pigeon classify</code> or <code>pigeon filter</code> .
subsample.txt	Output subsampling text report file.

Options	Description
<code>-s, --sub-sample-increment</code>	Specifies the number of reads between subsampling data points. (Default = 10000)
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of <code>stdout</code> .
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL] (Default = WARN)

`make-seurat` command: Generates the following output files required to run tertiary analysis using the [Seurat](#) software:

- barcodes.tsv
- genes.tsv
- matrix.mtx
- info.csv
- annotated.info.csv

### Usage

```
pigeon make-seurat [options] <classification.txt> -a <annotation GTF|XML> --dedup
<dedup fasta> --group <collapse group TXT>
```

### Example

```
pigeon make-seurat --dedup dedup.fasta --group
scisoseq.mapped_transcripts.collapse.group.txt
scisoseq_classification.filtered_lite_classification.txt -d output -a
absolutized.referenceset.xml
```

Required	Description
classification.txt	Text file containing transcript classifications. Could be generated by <code>pigeon classify</code> or <code>pigeon filter</code> .
<code>--dedup</code>	Specifies a molecule FASTA file generated by <code>isoseq3 groupdedup</code> or <code>isoseq3 dedup</code> .
<code>-g, --group</code>	Specifies an output groups text file generated by <code>isoseq collapse</code> .
<code>-a, --annotation</code>	Specifies reference annotations for Seurat gene matrix. May be a GTF/ GFF or Reference-Set XML file.

Input/output options	Description
<code>-o, --out-prefix</code>	Specifies a prefix for all non-matrix files. If <b>not</b> specified, the primary input classification file is used to determine the prefix. <b>Example:</b> <code>my_isoforms_classification.txt</code> input yields an output prefix of <code>my_isoforms</code> .

<b>Input/output options</b>	<b>Description</b>
<code>-d, --out-dir</code>	Specifies the destination directory for all output files. (Default = [ . ])

<b>Matrix options</b>	<b>Description</b>
<code>--keep-novel-genes</code>	Specifies that novel genes be kept in the matrix output.
<code>--keep-ribo-mito-genes</code>	Specifies that ribosomal and mitochondrial genes be kept in the matrix output.

<b>Options</b>	<b>Description</b>
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file</code>	Logs to a file, instead of stdout.
<code>--log-level</code>	Specifies the log level; values are [ TRACE, DEBUG, INFO, WARN, FATAL. ] (Default = WARN)

**primrose** The `primrose` tool analyzes the kinetic signatures of cytosine bases in CpG motifs to identify the presence of 5mC.

`primrose` uses a convolution neural network (CNN) to predict the methylation state (5mC) of each CpG in a HiFi read. Methylation is assumed to be symmetric between strands with output reported in the forward direction with respect to the HiFi read sequence. The output uses the `Mm` and `Ml` tags as defined in the SAM Format Optional Fields Specification. (See [here](#) for details.)

### Algorithm

The CNN is trained using invitro modified controls of methylated and unmethylated human DNA. The unmethylated control comprises a human shotgun library that has undergone whole genome amplification (WGA), a process that includes PCR, and therefore removes all modifications. The methylated control is generated by subjecting a human WGS library to invitro methylation using a CpG methyltransferase enzyme (*M. SssI*). Kinetic data, pulse width and inter-pulse distance, over a 16 base pair window for both the forward and reverse strand, is used as input to the CNN. The output of the CNN is a probability scale measure of whether the CpG is symmetrically 5mC-modified.

### Usage

```
primrose [options] <HiFi INPUT> <HiFi OUTPUT>
```

Required	Description
HiFi INPUT	Input BAM file or ConsensusReadSet XML file.
HiFi OUPUT	Output BAM file or ConsensusReadSet XML file.

Options	Description
<code>--keep-kinetics</code>	Specifies that the kinetics tracks (IPD and PulseWidth records) <code>fi</code> , <code>fp</code> , <code>fn</code> , <code>ri</code> , <code>rp</code> and <code>rn</code> are included in the output BAM file.
<code>--min-passes</code>	Specifies the minimum number of passes. (Default = 3)
<code>--model</code>	Specifies a path to a trained TensorFlow model directory, or to an exported ONNX model file.
<code>-h</code> , <code>--help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>-j</code> , <code>--num-threads</code>	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
<code>--log-file LOG_FILE</code>	Writes the log to file. Default (None) will write to <code>stderr</code> .
<code>--log-level</code>	Specifies the log level; values are [TRACE, DEBUG, INFO, WARNING, , FATAL.] (Default = WARNING)

### Example

```
primrose --log-level INFO movie.hifi.bam movie.hifi_5mc.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
<code>dataset_xml</code>	Input SubreadSet or ConsensusReadSet XML, which <b>must</b> contain an <code>sts.xml</code> resource for the full Run QC report set to be generated.
<code>-o OUTPUT_DIR</code>	Output report directory. (Default = Current working directory)

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

### Examples

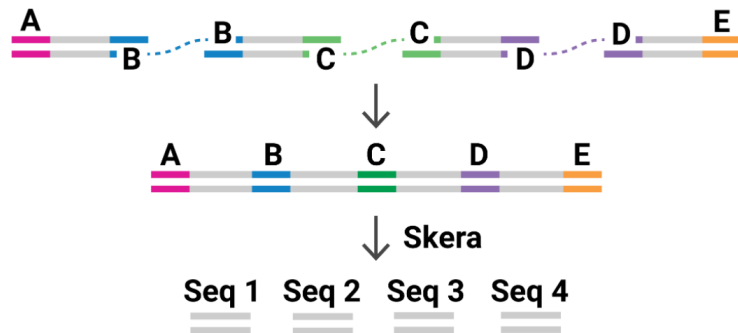
```
runqc-reports moviename.subreadset.xml
runqc-reports moviename.consensusreadset.xml
```

**skera** The `skera` tool splits arrayed HiFi reads at adapter positions, generating segmented reads (S-reads) which are the individual fragments. For each input HiFi read, the tool creates multiple BAM records, one for each fragment. An arrayed HiFi read can contain many fragments (or S-reads).

The `skera` tool also includes a command to reconstitute the original parent reads from input fragments.

### Segmented reads

An arrayed HiFi read is comprised of multiple fragments. The figure below displays an arrayed HiFi read with four fragments (Seq 1 through Seq 4).



### Segmented read BAM header

Arrayed HiFi reads have a `READTYPE` of `CCS` in the `DS` (Description) tag in the BAM header. After running `skera split`, segmented reads (S-reads) will have a `READTYPE` of `SEGMENT`. Additionally, a `SOURCE` key is added with a value of `CCS`.

#### Header `DS` tag for arrayed HiFi read:

```
DS:READTYPE=CCS;
```

#### Header `DS` tag for segmented read (S-Read):

```
DS:READTYPE=SEGMENT;SOURCE=CCS;
```

### Segmented read names

For segmented reads (S-reads), the base `QNAME` follows the CCS read conventions, while also appending the 0-based coordinate interval (`qStart`, `qEnd`) that represents its span within the arrayed HiFi read.

#### Default:

```
{movieName}/{holeNumber}/ccs/{qStart}_{qEnd}
```

### If the CCS read is split by strand:

```
{movieName}/{holeNumber}/ccs/fwd/{qStart}_{qEnd}
{movieName}/{holeNumber}/ccs/rev/{qStart}_{qEnd}
```

BAM tag	Type	Description	Example
di	Integer	Index of the read segment.	di:i:0
qs	Integer	qStart of the segment in the parent read.	qs:i:16
qe	Integer	qEnd of the segment in the parent read.	qe:i:450
dl	Integer	Leading segmentation adapter index.	dl:i:0
dr	Integer	Trailing segmentation adapter index.	dr:i:1
ds	Binary	Binary JSON file.	ds:b:10,21,23

### Notes:

- All tags of type `Integer` are 0-based.
- `qs` and `qe` tags are left open, right closed.
- `dl` and `dr` tags match the ordering of adapter sequences in the user input Adapter FASTA.
- The `ds` tag contains a number of fields used for supporting the `skera undo` command, which are **not** intended for general use.

`skera split` command: Splits sequences at adapter locations.

### Usage

```
skera split [options] <Input dataset> <ADAPTERS.fasta> <Output dataset>
```

Required	Description
Input dataset	Input HiFi reads in BAM/ConsensusReadSet XML format.
ADAPTERS.fasta	Segmentation adapters FASTA or AdapterSet XML file containing segmentation adapters. If not specified, the adapters specified in the XML metadata are used. Segmentation adapters <b>must</b> be ordered in the expected order of adapters in the reads. There should be one entry per adapter (forward or reverse-complement orientation) with no overlapping adapter sequences. Duplicate names or sequences are not allowed. <b>Example:</b> >A AGCTTACTTGTGAAGA >B ACTTGTAAGCTGTCTA >C ACTCTGTCAGGTCCGA >D ACCTCCTCCTCCAGAA >E >AACCGGACACACTTAG
Output dataset	Output S-reads in BAM/ConsensusReadSet XML format.

Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
-j, --num-threads	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
--log-file LOG_FILE	Writes the log to file. Default (None) will write to stderr.
--log-level	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL]. (Default = WARN)

`skera undo` command: Reconstitutes the original parent reads from input fragments (S-reads). **Note:** To run `skera undo`, **both** the `passing` and `non_passing` BAM files **must** be present in the same directory.

### Usage

`skera undo [options] <Input dataset> <Output dataset>`

Required	Description
Input dataset	<code>skera split</code> reads in BAM/ConsensusReadSet XML format.
Output dataset	Arrayed HiFi reads in BAM/ConsensusReadSet XML format.

Options	Description
-h, --help	Displays help information and exits.
--version	Displays program version number and exits.
-j, --num-threads	Specifies the number of threads to use when processing; 0 means autodetection. (Default = 0)
--log-file LOG_FILE	Writes the log to file. Default (None) will write to stderr.
--log-level	Specifies the log level; values are [TRACE, DEBUG, INFO, WARN, FATAL]. (Default = WARN)

### Input files for `skera split`

- \*.bam: Arrayed HiFi reads.
- <adapter>.fasta: A FASTA file containing segmentation adapters.

### Output files for `skera split`

- \*.bam: BAM file containing S-reads that were segmented from arrayed HiFi reads.
- \*.non\_passing.bam: BAM file containing HiFi reads that failed segmentation. Any arrayed HiFi read in which at least one segment is flanked by adapter sequences that are out of order (as specified by the segmentation adapter FASTA file) is output to the `*non_passing.bam` file to enable the `skera undo` command.  
**Note:** To run `skera undo`, **both** the `passing` and `non_passing` BAM files **must** be present in the same directory.
- \*.summary.json, \*summary.csv: Contains the following summary statistics:



- 
- **Reads:** The number of input arrayed HiFi reads.
  - **Segmented Reads (S-reads):** The number of generated S-reads.
  - **Mean Length of S-reads:** The mean read length of the generated S-reads.
  - **Percentage of Reads with Full Array:** The percentage of input reads containing all adapter sequences in the order listed in the segmentation adapter FASTA file.
  - **Mean Array Size:** The mean number of fragments (or S-reads) found in the input reads.
- \*.ligations.csv: Contains counts of adapter ligations found in arrayed HiFi reads. For each sequence found between two adapters, a ligation between two adapters is counted. The ligations are counted in the order of the adapters in the arrayed HiFi read (adapter\_1, adapter\_2).
  - \*.read\_lengths.csv: Contains the HiFi read length, S-Read length, and array size for each HiFi read.

### Examples

```
skera split <movie>.hifi_reads.bam adapters.fasta <movie>.skera.bam
```

```
skera undo <movie>.skera.bam <movie>.undo.bam
```

## 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
<code>-h, --help</code>	Displays help information and exits.
<code>--version</code>	Displays program version number and exits.
<code>--log-file LOG_FILE</code>	Writes the log to file. Default (None) will write to stdout.
<code>--log-level</code>	Specifies the log level; values are [DEBUG, INFO, WARNING, ERROR, CRITICAL] (Default = INFO)
<code>--debug</code>	Alias for setting the log level to DEBUG. (Default = False)
<code>--quiet</code>	Alias for setting the log level to CRITICAL to suppress output. (Default = False)
<code>--verbose, -v</code>	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 `pbcrumwell show-workflow-details` command, which is available for **all** applications.

### Examples

```
pbcrumwell show-workflow-details pb_demux_ccs
pbcrumwell show-workflow-details cromwell.workflows.pb_export_ccs
```

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

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

**Demultiplex Barcodes**    **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	<code>pb_demux_ccs.summary_csv</code>
Demultiplexed Datasets	<code>pb_demux_ccs.demuxed_files_datastore</code>
Unbarcoded Reads	<code>pb_demux_ccs.unbarcoded</code>

**Export Reads**    **Analysis application name:** cromwell.workflows.pb\_export\_ccs

**Entry point**

: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**

**Analysis application name:** cromwell.workflows.pb\_assembly\_hifi

**Entry point**

: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

**HiFi Mapping**    **Analysis application name:** cromwell.workflows.pb\_ccs\_subreads

**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

## HiFiViral SARS-CoV-2 Analysis

**Analysis application name:** cromwell.workflows.pb\_sars\_cov2\_kit

### Entry points

```
: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
```

### 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

**Analysis application name:** cromwell.workflows.pb\_isoseq3\_cconly

### 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_cconly.collapse_fastq
Collapsed Filtered Isoforms GFF	pb_isoseq3_cconly.collapse_gff
Group TXT	pb_isoseq3_cconly.collapse_group
Abundance TXT	pb_isoseq3_cconly.collapse_abundance
Read Stat TXT	pb_isoseq3_cconly.collapse_readstat
Collapsed Isoforms Abundance TXT (Files are numbered consecutively, 1 for each barcoded sample.)	pb_isoseq3_cconly.collapse_abundance Separate clusters, one per barcoded sample.
Collapsed Isoforms Abundance TXT (Files are numbered consecutively, 1 for each barcoded sample.)	pb_isoseq3_cconly.collapse_abundance Pooled clusters, one per barcoded sample.
High-Quality Transcripts	pb_isoseq3_cconly.hq_fastq
Low-Quality Transcripts	pb_isoseq3_cconly.lq_fastq
High-Quality Transcripts Counts (Files are numbered consecutively, 1 for each barcoded sample.)	pb_isoseq3_cconly.barcode_overview_report Separate clusters, one per barcoded sample.
High-Quality Transcripts Counts (Files are numbered consecutively, 1 for each barcoded sample.)	pb_isoseq3_cconly.barcode_overview_report Pooled clusters, one per barcoded sample.
CCS reads FASTQ	pb_isoseq3_cconly.ccs_fastq_zip
Full-length CCS reads	pb_isoseq3._cconly.flnc_bam
Polished Report	pb_isoseq3._cconly.polish_report_csv
Cluster Report	pb_isoseq3._cconly.report_isoseq

### Mark PCR Duplicates

**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  
Genome  
Analysis****Analysis application name:** cromwell.workflows.pb\_microbial\_analysis**Entry point**

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

**Key output files**

File name	Datastore SourceId
Final Polished Assembly	pb_microbial_analysis.assembly_fasta
Final Polished Assembly Index	pb_microbial_analysis.assembly_fasta.fai
Final Polished Assembly for NCBI	pb_microbial_analysis.ncbi_fasta
Mapped BAM	pb_microbial_analysis.mapped
Target sequences used to produce the Mapped BAM	pb_microbial_analysis.mapped_target_fasta
Target sequence Index used to produce the Mapped BAM	pb_microbial_analysis.mapped_target_fasta_fai
List of Circular Contigs	pb_microbial_analysis.circular_list
Coverage Summary	pb_microbial_analysis.coverage_gff
Coverage Report	pb_microbial_analysis.report_coverage
Mapping Statistics Report	pb_microbial_analysis.report_mapping_stats
Mapped BAM Datastore	pb_microbial_analysis.mapped_bam_datastore
Polished Assembly Report	pb_microbial_analysis.report_polished_assembly
Full Kinetics Summary	pb_microbial_analysis.basemods_gff
IPD Ratios	pb_microbial_analysis.basemods_csv
Motifs and Modifications	pb_microbial_analysis.motifs_csv
Motifs and Modifications	pb_microbial_analysis.motifs_gff

**Read  
Segmentation****Analysis application name:** cromwell.workflows.pb\_segment\_reads**Entry points**

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

:id: Barcode
:name: MAS-Seq Adapters
:fileTypeId: PacBio.DataSet.BarcodeSet
```

**Key output files**

File name	Datastore SourceId
Input Adapters	pb_segment_reads.adapters_fasta
Report Read Segmentation	pb_segment_reads.report_read_segmentation
Non-Passing Reads	pb_segment_reads.non_passing_bam

File name	Datastore SourceId
Segmented Reads	pb_segment_reads.segmented_reads
Segmented Reads BAM	pb_segment_reads.segmented_reads_bam

**Read Segmentation and Single-Cell Iso-Seq Analysis**

**Analysis application name:**

cromwell.workflows.pb\_segment\_reads\_and\_sc\_isoseq

**Entry points**

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

:id: eid_ref_dataset
:name: Reference Genome
:fileTypeId: PacBio.DataSet.ReferenceSet

:id: eid_barcode
:name: MAS-Seq Adapters
:fileTypeId: PacBio.DataSet.BarcodeSet

:id: eid_barcode_2
:name: Single-Cell Iso-Seq Primers
:fileTypeId: PacBio.DataSet.BarcodeSet
```

**Key output files**

File name	Datastore SourceId
Input Adapters	pb_segment_reads_and_sc_isoseq.adapters_fasta
Report Read Segmentation	pb_segment_reads_and_sc_isoseq.report_read_segmentation
Barcode statistics	scisoseq.bcstats_report_tsv
Transcript classifications	scisoseq.classification_txt
Transcript classification file, after SQANTI filtering criteria	scisoseq_classification.filtered_lite_classification.txt
Transcript junction file	scisoseq_junctions.txt
Transcript junction file, after SQANTI filtering criteria	scisoseq_classification.filtered_lite_junctions.txt
Unique transcripts, sorted GFF file	scisoseq_transcripts.sorted.gff
Unique transcripts, after SQANTI filtering criteria, GFF file	scisoseq_transcripts.sorted.filtered_lite.gff
Segmented reads, unmapped BAM file	segmented.bam
Reads that did not pass segmentation, unmapped BAM file	segmented.non_passing.bam
Read segmentation report	read_segmentation.report.json
Deduplicated reads mapped BAM file and index file	scisoseq.mapped.bam, scisoseq.mapped.bam.bai
Deduplicated reads, unmapped BAM file	scisoseq.5p--3p.tagged.refined.corrected.sorted.dedup.bam



File name	Datastore SourceId
Single-cell isoform and gene matrix, gzipped	scisoseq.seurat_info.tar.gz

**Single-Cell  
Iso-Seq  
Analysis**

**Analysis application name:** cromwell.workflows.pb\_sc\_isoseq

**Entry points**

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

:id: eid_ref_dataset
:name: Reference Genome
:fileTypeId: PacBio.DataSet.ReferenceSet

:id: eid_barcode
:name: Single-Cell Iso-Seq Primers
:fileTypeId: PacBio.DataSet.BarcodeSet
```

**Key output files**

File name	Datastore SourceId
Barcode statistics	scisoseq.bcstats_report_tsv
Transcript classifications	scisoseq.classification_txt
Transcript classification file, after SQANTI filtering criteria	scisoseq_classification.filtered_lite_classification.txt
Transcript junction file	scisoseq_junctions.txt
Transcript junction file, after SQANTI filtering criteria	scisoseq_classification.filtered_lite_junctions.txt
Unique transcripts, sorted GFF file	scisoseq_transcripts.sorted.gff
Unique transcripts, after SQANTI filtering criteria, GFF file	scisoseq_transcripts.sorted.filtered_lite.gff
Segmented reads, unmapped BAM file	segmented.bam
Reads that did not pass segmentation, unmapped BAM file	segmented.non_passing.bam
Read segmentation report	read_segmentation.report.json
Deduplicated reads mapped BAM file and index file	scisoseq.mapped.bam, scisoseq.mapped.bam.bai
Deduplicated reads, unmapped BAM file	scisoseq.5p--3p.tagged.refined.corrected.sorted.dedup.bam
Single-cell isoform and gene matrix, gzipped	scisoseq.seurat_info.tar.gz

## Structural Variant Calling

**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 Ultra-Low Adapters

**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).

---

**5mC CpG  
Detection****Analysis application name:** cromwell.workflows.pb\_detect\_methyl**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
HiFi reads with 5mC Calls	pb_detect_methyl.ccsbam

---

## 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.
- ipython**
  - An interactive shell for using the PacBio 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](https://github.com/dfguan/purge_dups) for details.
- python**
  - An object-oriented programming language.
  - See <https://www.python.org/> for details.
- samtools, BCFtools**
  - A set of programs for interacting with high-throughput sequencing data in SAM/BAM/CRAM/VCF/BCF2 formats.
  - See <http://www.htslib.org/> for details.

---

## Appendix C - Microbial Genome Analysis 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 workflow also performs base modification analysis of the assembled genome.

The Microbial Genome Analysis application:

- Includes chromosomal- and plasmid-level *de novo* genome assembly, circularization, polishing, and rotation of the origin of replication for each circular contig.
- Performs base modification detection (m4C and m6A).
- Facilitates assembly of larger genomes (yeast) as well.
- Accepts HiFi read data (BAM format) as input.

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

**Chromosomal 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. Chromosomal stage parameters are prefixed with:  
`ipa2_advanced_options_chrom`. In the SMRT Link interface for the Microbial Genome Analysis application, this corresponds to the **Advanced Assembly Options for chromosomal stage** field.
2. Plasmid stage parameters are prefixed with:  
`ipa2_advanced_options_plasmid`. In the SMRT Link interface for the Microbial Genome Analysis application, this corresponds to the **Advanced Assembly Options for plasmid stage** field.

---

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 44](#) for details.