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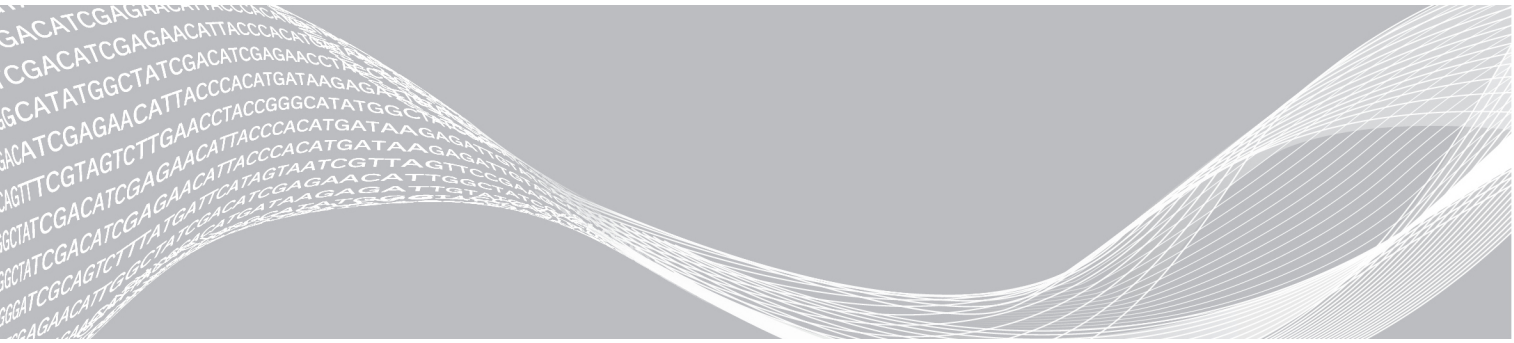
bcl2fastq2 Conversion v2.19

User Guide



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Introduction

The Illumina sequencing instruments generate per-cycle base call (BCL) files at the end of the sequencing run. A majority of analysis applications use per-read FASTQ files as input for analysis. You can use the bcl2fastq2 Conversion Software v2.19 to convert base call (BCL) files from a sequencing run into FASTQ files.

Use this guide to install the bcl2fastq2 Conversion Software and run the BCL conversion and demultiplexing process.

Supported Instruments

The bcl2fastq2 Conversion Software supports the following instruments:

- MiniSeq
- MiSeq
- NextSeq 500, 550
- HiSeq X
- HiSeq 2000, 2500, 3000, 4000
- NovaSeq 5000, 6000

If your Illumina sequencing system runs an earlier software version of Real-Time Analysis (RTA) than v1.18.54 and you want to convert BCL to FASTQ, install bcl2fastq v1.8.4, and refer to the *bcl2fastq Conversion User Guide Version v1.8.4 (part # 15038058)* for instructions.

BCL Conversion and Demultiplexing Directory

The bcl2fastq2 Conversion Software performs BCL conversion and demultiplexing in a single step. By default, the software puts the resulting demultiplexed compressed FASTQ files in `<run folder>/Data/Intensities/BaseCalls`.

The software puts reads with undetermined indexes in files that begin with `Undetermined_S0_`. If unindexed samples are included in a lane with indexed information, the software exits with an error (missing a barcode).

If the Sample_Project column is specified for a sample in the sample sheet, the FASTQ files for that sample are placed in `<run folder>/Data/Intensities/BaseCalls/<Project>`.

Multiple samples can use the same project directory. If the Sample_ID and Sample_Name columns are specified but do not match, the FASTQ files are placed in an additional sub-directory called `<SampleId>` with files named using the Sample_Name value.

BCL to FASTQ Conversion Process

The bcl2fastq2 Conversion Software converts the base calls in the per-cycle BCL files to the per-read FASTQ format. As an option, the software can trim adapters and remove Unique Molecular Identifier (UMI) bases from reads.

Adapter Trimming—The bcl2fastq2 Conversion Software checks whether a read extends past the sample DNA insert and into the adapter sequence. The software uses an approximate string matching algorithm to identify all or part of the adapter, and treats the insertions and deletions as a single mismatch. If an adapter sequence is detected, base calls matching the adapter and beyond the match are masked or removed in the FASTQ file.

Unique Molecular Indentifiers (UMIs) Removal—UMIs are random k-mers attached to the genomic DNA before polymerase chain reaction (PCR) amplification. After the UMI is amplified with amplicons, the software can retrieve these bases and place them into the read name in the FASTQ files. Also, when the TrimUMI sample sheet setting is active, the software can remove the bases from the reads.

Demultiplexing—First, the software reorganizes the FASTQ files based on the index sequencing information. For best practices, avoid choosing indexes that differ by fewer than 3 bases during sample preparation. After generating the FASTQ files, the software generates the statistics and reports for the demultiplexed FASTQ files. The software also recalculates the base calling analysis statistics and store the statistics in the InterOp folder. You can view the statistics with the Sequencing Analysis Viewer (SAV) software from Illumina.

Output Files

- FASTQ Files
- InterOp Files
- ConversionStats File
- DemultiplexingStats File
- Adapter Trimming File
- FastqSummary and DemuxSummary
- HTML Reports
- JSON File

Install bcl2fastq2 Conversion Software

You can download the bcl2fastq2 Conversion Software from the Downloads page on the Illumina website.

For installation requirements, see *Appendix: Installation Requirements* on page 26.

Install from RPM Package

You need to have access the root system to install.

1 To install the RPM file, use the following command line:

```
yum install -y <rpm package-name>
```

The starting point for the bcl2fastq converter is the binary executable `/usr/local/bin/bcl2fastq`.

2 To install the RPM package in a user specified location, use the following command line:

```
rpm --install --prefix <user specified directory>
   <rpm package-name>
```

Install from Source

For installation, the directory locations are specified with the following environment variables:

Variables	Description
SOURCE	Location of the bcl2fastq2 source code
BUILD	Location of the build directory
INSTALL_DIR	Location where the executable is installed

For example, the environment variables can be set as:

```
export TMP=/tmp
export SOURCE=${TMP}/bcl2fastq
export BUILD=${TMP}/bcl2fastq2-v2.19.x-build
export INSTALL_DIR=/usr/local/bcl2fastq2-v2.19.x
```

The build directory must be different from the source directory.

Follow these steps to install from source:

1 Decompress and extract the source code.

```
cd ${TMP}
tar -xvzf bcl2fastq2-v2.19.x.tar.gz
```

This command populates the directory ${TMP}/bcl2fastq.

2 Configure the build using the following commands:

```
mkdir ${BUILD}
cd ${BUILD}
chmod ugo+x ${SOURCE}/src/configure
chmod ugo+x ${SOURCE}/src/cmake/bootstrap/installCmake.sh
${SOURCE}/src/configure --prefix=${INSTALL_DIR}
```

The first two commands create a build directory to work from. The next two lines ensure necessary files can be executed. Executing the final configure command populates the ${BUILD} directory with that necessary files needed to build bcl2fastq2 in step 3 . The --prefix parameter provides the absolute path to the installation directory. Make sure you have write permission to the ${INSTALL_DIR} directory. The ${BUILD} directory will be created.

3 Build and install the package using the following commands:

```
cd ${BUILD}
make
make install
```

Depending on the ${INSTALL_DIR} directory, you may need root privilege.

BCL Conversion Input Files

After sequencing, the instruments generate a BaseCalls directory, which contains the base calls files (BCL), for demultiplexing.

For demultiplexing, the bcl2fastq2 Conversion Software requires the following input files:

Instrument	Input Files
MiSeq and HiSeq 2000/2500	• BCL Files (*.bcl.gz) • STATS Files • FILTER Files • Position Files • RunInfo Files • Config Files • Sample Sheet Files (optional)
MiniSeq and NextSeq 500/550	• BCL Files (*bcl.bgzf) • BCI Files • FILTER Files • Position Files • RunInfo Files • Sample Sheet Files (optional)

Instrument	Input Files
HiSeq X and HiSeq 3000/4000	• BCL Files (*.bcl.gz) • FILTER Files • Position Files • RunInfo Files • Sample Sheet Files (optional)
NovaSeq	• CBCL Files (*.cbcl) • FILTER Files (*.filter) • Position Files (s.locs) • Runinfo FIles (Runinfo.xml) • Samples Sheet Files (SampleSheet.csv, optional)

BCL Conversion Input Files Diagram

Figure 1 BCL Conversion Input Files from the MiSeq or HiSeq 2000/2500 System

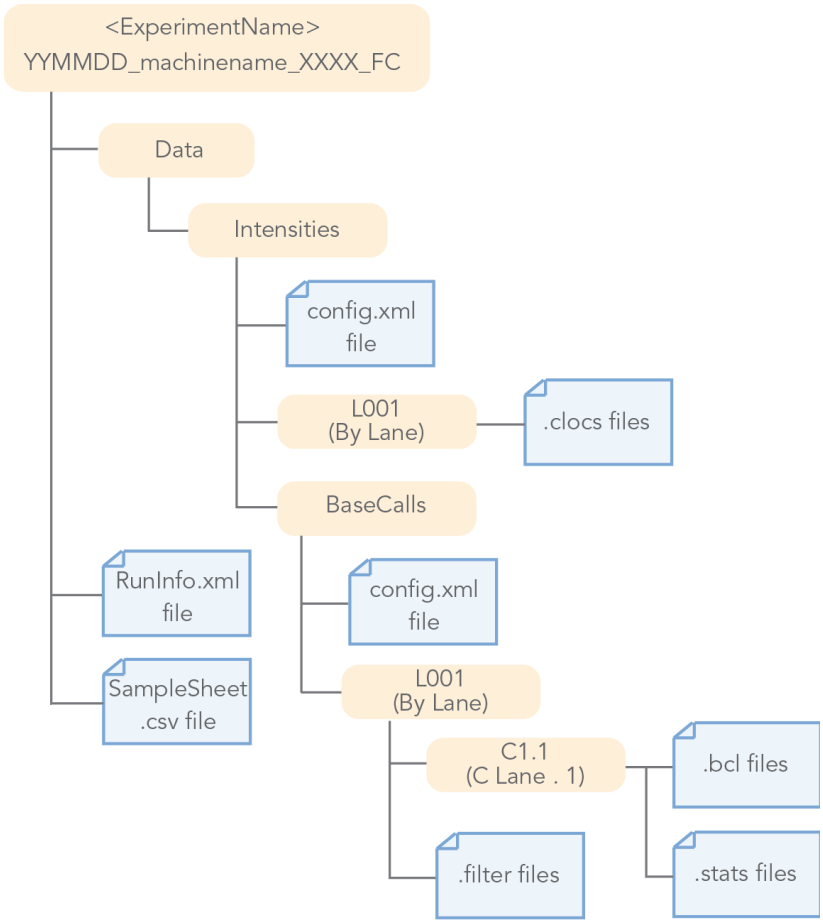


Figure 2 BCL Conversion Input Files from the MiniSeq or NextSeq System

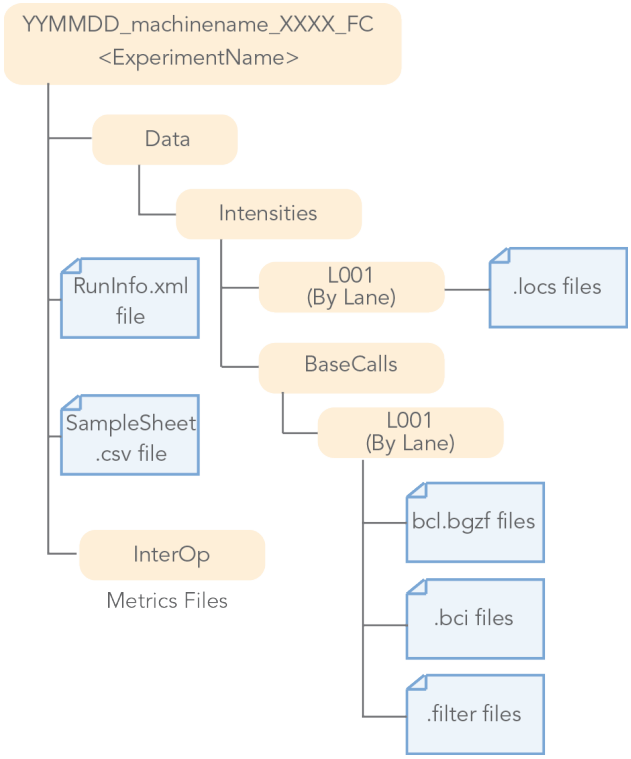


Figure 3 BCL Conversion Input Files from the HiSeq X System

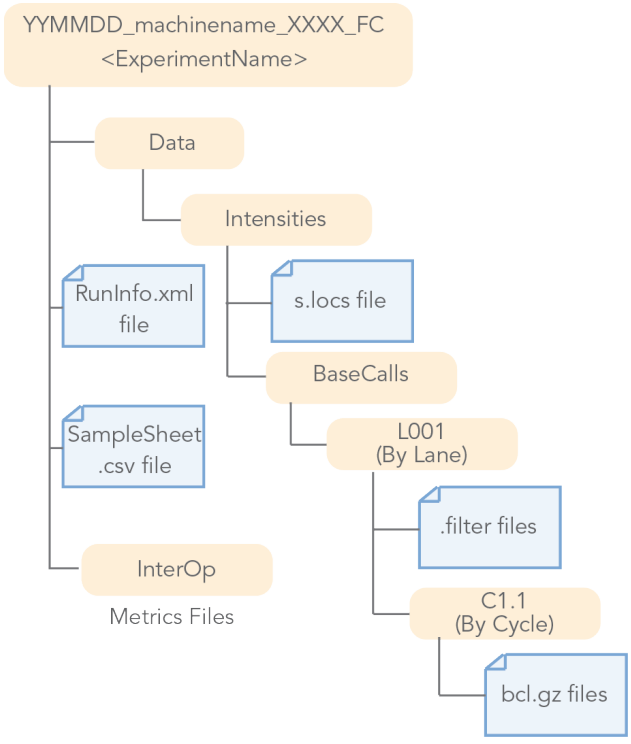
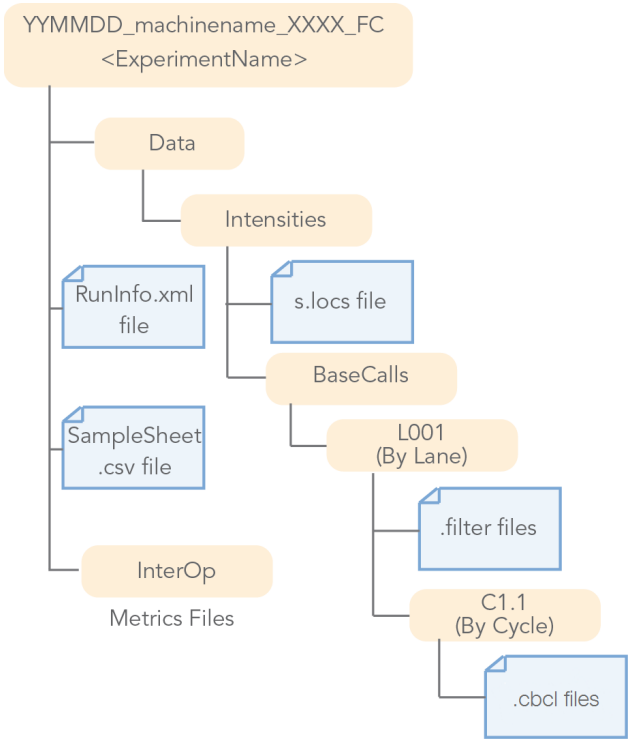


Figure 4 BCL Conversion Input Files from the NovaSeq System



Folder and File Naming

The top-level run folder name is generated using 3 fields to identify the <ExperimentName>, separated by underscores.

The software generates the top-level run folder using 3 fields separated by underscores to identify the <ExperimentName>.

Example:

```
YYMMDD_machinename_NNNN
```

For best practices, do not deviate from the run folder naming convention because doing so can cause the software to stop.

- The first field is a six-digit number (YYMMDD) specifying the date of the run.
- The second field specifies the name of the sequencing machine. The field can consist of any combination of upper or lower case letters, digits, or hyphens, but it ***cannot*** contain any other characters or underscore.
- The third field is a four-digit number that specifies the experiment ID on that instrument. Each instrument supplies a series of consecutively numbered experiment IDs from the on-board sample tracking database or a LIMS.

For best practices, we recommend that you create unique names for the experiment or sample IDs for each instrument to avoid naming conflicts.

For example, a run folder named **150108_instrument1_3147** indicates that the experiment ID is 3147; the run is on instrument 1, and the date is on January 8, 2015 (YYMMDD). The date and instrument name specify a unique run folder for any number of instruments.

Also, you can view the flow cell number in the run folder name.

Example:

```
YYMMDD_machinename_NNNN_FCYYY
```

When you publish the data to a public database, we recommend that you use a prefix for each instrument with the identity of the sequencing center.

BCL Files

The BCL files are compressed with the gzip (*.gz) or the blocked GNU zip (*.bgzf) format.

The BaseCalls directory contains the BCL files. The NextSeq and MiniSeq files are located in the following directory:

```
Data/Intensities/BaseCalls/L<lane>
```

You can locate the HiSeq and MiSeq files in the following directory:

```
Data/Intensities/BaseCalls/L<lane>/C<Cycle>.1
```

Table 1 BCL File Format

Bytes	Description	Data type
Bytes 0–3	Number N of cluster	Unsigned 32 bits integer
Bytes 4–(N+3) **N**—Cluster index	Bits 0–1 are the bases, [A, C, G, T] for [0, 1, 2, 3]: bits 2–7 are shifted by 2 bits and contain the quality score. All bits with 0 in a byte is reserved for no call.	Unsigned 8 bits integer

CBCL Files

The BCL data are aggregated and written out in the CBCL format when aggregation is on (the current aggregation scheme is per lane/surface). The CBCL file format is as follows:

Table 2 CBCL File Format

CBCL File Header		
Bytes/Field	Description	Data Type
Bytes 0 - 1	Version number, current version is 1	unsigned 16 bits little endian integer
Bytes 2 - 5	Header size	unsigned 32 bits little endian integer
Byte 6	Number of bits per basecall	unsigned
Byte 7	Number of bits per q-score	unsigned
q-val mapping info		
Bytes 0-3	Number of bins (B), zero indicates no mapping	
B pairs of 4 byte values (if B > 0)	{**from**, **to**}, {**from**, **to**}, {**from**, **to**} … **from**: quality score bin **to**: quality score	
Number of tile records		unsigned 32bits little endian integer
gzip virtual file offsets, one record per tile		
Bytes 0-3: tile number		
Bytes 4-7	Number of clusters that were written into the current block (required due to bit-packed q-scores)	unsigned 32 bit integer

Bytes 8-11	Uncompressed block size of the tile data (useful for sanity check when excluding non-PF clusters)	unsigned 32 bit integer
Bytes 12-15	Compressed block size of the tile data	unsigned 32 bit integer
non-PF clusters excluded flag	1: non-PF clusters are excluded 0: non-PF clusters are included	
CBCL File Content		
N blocks of gzip files, where N is the number of tiles. Each block consists of C number of basecall, quality score pairs where C is the number of clusters for the given tile. Each basecall, quality score pair has the following format (assuming 2 bits are used for the basecalls): Bits 0-1: Basecalls (respectively [A, C, G, T] for [00, 01, 10, 11]) Bits 2 and up: Quality score (unsigned Q bit little endian integer where Q is the number of bits per q-score). For a two bit quality score, this is two clusters per byte where the bottom 4 bits are the first cluster and the higher 4 bits are the second cluster.		

BCI Files

The BCI (*.bci) files contain one record per tile for the sequencing run in binary format. You can locate these files from the following directory:

```
<run directory>/Data/Intensities/BaseCalls/L<lane>
```

Table 3 BCI File Format

Bytes	Description
Bytes 0–3	Tile number
Bytes 4–7	Number of clusters in the tile

STATS Files

The STATS file (*.stats) is a binary file that contains base calling statistics. You can locate these files from the following directory:

```
Data/Intensities/BaseCalls/L00<lane>/C<cycle>.1
```

Table 4 Stats File Format

Start	Description	Data Type
Byte 0	Cycle number	integer
Byte 4	Average Cycle Intensity	double
Byte 12	Average intensity for A over all clusters with intensity for A	double
Byte 20	Average intensity for C over all clusters with intensity for C	double
Byte 28	Average intensity for G over all clusters with intensity for G	double
Byte 36	Average intensity for T over all clusters with intensity for T	double
Byte 44	Average intensity for A over clusters with base call A	double
Byte 52	Average intensity for C over clusters with base call C	double
Byte 60	Average intensity for G over clusters with base call G	double
Byte 68	Average intensity for T over clusters with base call T	double
Byte 76	Number of clusters with base call A	integer
Byte 80	Number of clusters with base call C	integer
Byte 84	Number of clusters with base call G	integer

Start	Description	Data Type
Byte 88	Number of clusters with base call T	integer
Byte 92	Number of clusters with base call X	integer
Byte 96	Number of clusters with intensity for A	integer
Byte 100	Number of clusters with intensity for C	integer
Byte 104	Number of clusters with intensity for G	integer
Byte 108	Number of clusters with intensity for T	integer

FILTER Files

The FILTER file (*.filter) is a binary file that contains the filter results. You can locate these files from the following directory:

```
Data/Intensities/BaseCalls/L<lane>
```

Table 5 Filter File Format

Bytes	Description
Bytes 0–3	Zero value (for backwards compatibility)
Bytes 4–7	Filter format version number
Bytes 8–11	Number of clusters
Bytes 12–(N+11) N — cluster number	Unsigned 8 bits integer Bit 0 is pass or failed filter

CONTROL Files

CONTROL files (*.control) are deprecated as of bcl2fastq v2.19 and are no longer used by the software.

CONFIG Files

NOTE

The CONFIG files are only created on RTA 1 systems (MiSeq and HiSeq 2500). They are not produced or expected on newer platforms.

The CONFIG (*config.xml) file records information specific to the generation of the subfolders. The file contains a tag-value list that describes the cycle-image folders used to generate each folder of intensity and sequence files. You can locate the file from the following directory:

```
<run directory>/Data/Intensities/
```

The other CONFIG (*config.xml) file is in the BaseCalls directory, which contains the meta-information on the base caller runs. You can locate the file from the following directory:

```
<run directory>/Data/Intensities/BaseCalls/
```

Position Files

The BCL to FASTQ converter can use different types of position files.

The LOCS (*.locs) file is a binary file that contains the cluster positions. Additionally, the *.clocs files are compressed versions of LOCS files.

The *_pos.txt files are text-based files with 2 columns and a number of rows equal to the number of clusters. The first column is the X-coordinate and the second column is the Y-coordinate. Each line has a <cr><lf> at the end.

You can locate these files in the following directory:
`Data/Intensities/L<lane>`

RunInfo File

The RunInfo.xml file is located at the top-level run folder `<run directory>`. The file contains information on the run, flow cell, and instrument IDs, date and read structure. Also, the file provides the number of reads, the number of cycles per read, and the index reads.

Sample Sheet

The sample sheet (*SampleSheet.csv) file provides information on the relationship between samples and indexes during library creation. The sample sheet is optional and the default location is the top-level run folder. You can use the --sample-sheet command line option to specify any CSV file in any location. When a sample sheet is not provided, all reads are assigned to the default sample `Undetermined_S0`, which includes one file per lane per read.

Settings Section

The bcl2fastq2 Conversion Software uses the Settings section of the SampleSheet to specify adapter trimming, UMI, and index-fastq options..

Table 6 Adapter Specifications

Setting	Description
`Adapter` or `TrimAdapter`	The adapter sequence to be trimmed. If an AdapterRead2 is provided, this sequence is only used to trim Read 1. To trim two or more adapters, separate the sequences by a plus sign (+). The plus sign between the adapters signifies that these are independent adapters and that they need to be assessed for trimming independently for each read.
`AdapterRead2` or `TrimAdapterRead2`	The adapter sequence to be trimmed in Read 2. If not provided, the same sequence specified in Adapter is used. To trim two or more adapters, separate the sequences by a plus sign (+). The plus sign between the adapters signifies that these are independent adapters and that they need to be assessed for trimming independently for each read.
`MaskAdapter`	The adapter sequence to be masked rather than trimmed. If MaskAdapterRead2 is provided, this sequence is only used to mask Read 1.
`MaskAdapterRead2`	The adapter sequence to be masked in Read 2. If not provided, the same sequence specified in MaskAdapter is used.
`FindAdaptersWithIndels`	1 (default) or 0. If 1 (true), an approximate string matching algorithm is used to identify the adapter, treating insertions and deletions as a single mismatch (Myers 1999, J.ACM). If 0 (false), a sliding window algorithm is used, in which insertions and deletions of bases inside the adapter sequence is not tolerated.

Table 7 Cycle and Tile Specifications

Setting	Description
`Read1EndWithCycle`	The last cycle to use for Read 1.
`Read2EndWithCycle`	The last cycle to use for Read 2.
`Read1StartFromCycle`	The first cycle to use for Read 1.
`Read2StartFromCycle`	The first cycle to use for Read 2.
`Read1UMILength`	The length of the UMI used for Read 1.
`Read2UMILength`	The length of the UMI used for Read 2.

Setting	Description
Read1UMIStartFromCycle	The first cycle to use for UMI in Read 1. The cycle index is absolute and not affected by Read1StartFromCycle. The software supports UMIs only at the beginning or end of reads. This sample sheet setting must be used in conjunction with the Read1UMILength sample sheet setting or it will be ignored.
Read2UMIStartFromCycle	The first cycle to use for UMI in Read 2. The cycle index is absolute and not affected by Read2StartFromCycle. The software currently supports UMIs only at the beginning or end of reads. This sample sheet setting must be used in conjunction with the Read2UMILength sample sheet setting or it will be ignored.
TrimUMI	0 (default) or 1 (true). When TrimUMI setting is set to 1, the software trims the UMI bases from Read 1 and Read 2.
ExcludeTiles	Tiles to exclude. Separate tiles using a plus sign [+], or specified as a range with a hyphen [-]. For example, ExcludeTiles,1101+2201+1301-1306 means skip tiles 1101, 2201, and 1301 through 1306.
ExcludeTilesLaneX	Tiles to exclude for Lane X. For example, ExcludeTilesLane6,1101-1108 means skip tiles 1101 through 1108 for lane 6 only.

Table 8 FASTQ Specifications

Setting	Description
CreateFastqForIndexReads	0 (default) or 1. If 1 (true), generate FASTQ files for index reads. Normally, these FASTQ files are not needed, because demultiplexing is carried out automatically based on the sample sheet. Also, the index sequence is already placed in the sequence identifiers in the FASTQ files. Generating FASTQ files is based on the following: • The index read masks are specified from the --use-bases-mask option. • The RunInfo.xml file when the --use-bases-mask option is not used.
ReverseComplement	0 (default) or 1. If 1 (true), all reads are reverse complemented as they are written to FASTQ files. This step is necessary in certain unusual cases (eg processing of mate-pair data using BWA, which expects paired-end data).

Data Section

The bcl2fastq2 Conversion Software uses the information in the columns of the Data section.

Column	Description
Lane	When specified, the software generates FASTQ files for only the samples with the specified lane number.
Sample_ID	The sample ID. Do not use "all" or "unknown" as the sample ID. If either of these is used as the name, the sample will be omitted from the report.
Sample_Name	The sample name. Note: Do not use "all" or "undetermined" as the sample name. If either of these is used as the name, the sample will be omitted from the report.
Sample_Project	The sample project name. The software creates a directory with the specified sample project name and stores the FASTQ files there. You can use multiple samples in the same project. Note: Do not use "all" or "default" as the sample project name. If either of these is used as the name, the sample will be omitted from the report.
index	The index sequence.
index2	The index sequence for index 2.

If the Sample_ID and Sample_Name columns do not match, the FASTQ files are placed in an additional sub-directory called <SampleId>.

You can use alphanumeric characters, hyphens [-], and underscores [_] for the Sample_Project, Sample_ID, and Sample_Name. Sample_ID, Sample_Name, and Sample_Project field entries in the sample sheet cannot contain illegal characters that are not allowed by some file systems. Examples of common characters that are not allowed are the space character and the following: ?()[]/\=+<>:;"',*^| &.

Sample Sheet Demultiplexing Scenarios

The Illumina Experiment Manager performs the following for sample sheet BCL conversion and demultiplexing:

- All reads are placed in the `Undetermined_S0` FASTQ files when there is no sample sheet.
- All reads are placed in the `Undetermined_S0` FASTQ files when there is a sample sheet but no data section.
- All reads are placed in the sample FASTQ file as defined in the sample sheet when there is a sample sheet and one sample with no indexes.
- When there is a sample sheet and the samples have indexes, the software performs the following:
  - Reads without a matching index are placed in the default `Undetermined_S0` FASTQ files.
  - Reads with a valid index are placed in the sample FASTQ file as defined in the sample sheet.

For each sample, there is one file per lane per read number when reads exist for that sample, lane, and read number.

NOTE

When the Lane column of the sample sheet Data section is populated, only those lanes are converted. When the Lane column is not used, all lanes are converted.

Create a Sample Sheet with IEM

The Illumina Experiment Manager (IEM) software helps you create and edit sample sheets for Illumina sequencers and analysis software. You can use IEM to create sample sheets for any Illumina sequencer.

You can download IEM at support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html.

View the Illumina Experiment Manager User Guide for creating a sample sheet.

Run BCL Conversion and Demultiplexing

Use the following command to run the bcl2fastq2 Conversion Software :

```
nohup /usr/local/bin/bcl2fastq [options]
```

An example of a command with options:

```
nohup /usr/local/bin/bcl2fastq --runfolder-dir <RunFolder>
   --output-dir <BaseCalls>
```

This command produces a set of FASTQ files in the BaseCalls directory. Reads with an unresolved or erroneous index are placed in the `Undetermined_S0` FASTQ files. By default, `--runfolder-dir` is the current directory and `--output-dir` is the Data/Intensities/BaseCalls sub-directory of the run folder.

BCL2FASTQ Options

The main command line options are the `--runfolder-dir` and `--output-dir`. For command line options that have a corresponding sample sheet setting, the value passed on the command line overwrites the value found in the sample sheet.

Table 9 Main Options

Option	Description
`-R, --runfolder-dir`	Path to run folder directory Default: ./
`-o, --output-dir`	Path to demultiplexed output Default: <runfolder-dir>/Data/Intensities/BaseCalls/

You can use the following advanced options for non-default settings or for customized settings.

Table 10 Directory Options

Option	Description
`-i, --input-dir`	Path to input directory Default: <runfolder-dir>/Data/Intensities/BaseCalls/
`--sample-sheet`	Path to sample sheet, so you can specify the location and name of the sample sheet, if different from default. Default: <runfolder-dir>/SampleSheet.csv

The following directory options and thread control options provide more control of the conversion process, but are not needed for standard usage.

Table 11 Additional Directory Options

Option	Description
`--intensities-dir`	Path to intensities directory If intensities directory is specified, then the input directory must also be specified. Default: <input-dir>/../
`--interop-dir`	Path to demultiplexing statistics directory Default: <runfolder-dir>/InterOp/
`--stats-dir`	Path to human-readable demultiplexing statistics directory Default: <output-dir>/Stats/
`--reports-dir`	Path to reporting directory Default: <output-dir>/Reports/

For processing, if your computing platform supports threading, the software manages the threads by the following defaults:

- 4 threads for reading the data
- 4 threads for writing the data
- 20% for demultiplexing data
- 100% for processing demultiplexed data

The file i/o threads spend most of their time sleeping, and so take little processing time. The processing of demultiplexed data is allocated 1 thread per CPU to make sure that there are no idle CPUs, resulting in more threads than CPUs by default. You can use the following options to provide control on threading. If, for example, you share your computing resources with colleagues and wish to limit your usage, these options are useful.

Table 12 Processing Options

Option	Description
`-r, --loading-threads`	Number of threads used for loading BCL data. Default depends on architecture.
`-p, --processing-threads`	Number of threads used for processing demultiplexed data. Default depends on architecture.
`-w, --writing-threads`	Number of threads used for writing FASTQ data. This number should not be set higher than number of samples. Default depends on architecture.

If you want to use these options to assign multiple threads, consider the following:

- The most CPU demanding stage is the processing step (-p option). Assign this step the most threads.
- Reading and writing stages are lightweight and do not need many threads. This consideration is especially important for a local hard drive where too many threads mean too many parallel read write actions giving suboptimal performance.
- Use one thread per CPU core plus a little more to supply CPU with work. This method prevents CPUs being idle due to a thread being blocked while waiting for another thread.
- The number of threads depends on the data. If you specify more writing threads than samples, the extra threads do no work but can cost time due to context switching.

Table 13 Behavioral Options

Option	Description
`--adapter-stringency`	The minimum match rate that would trigger the masking or trimming process. This value is calculated as MatchCount / (MatchCount + MismatchCount) and ranges from 0 to 1, but it is not recommended to use any value < 0.5, as this value would introduce too many false positives. The default value for this parameter is 0.9, meaning that only reads with > 90% sequence identity with the adapter are trimmed. Default: 0.9
`--barcode-mismatches`	Number of allowed mismatches per index Multiple entries, comma delimited allowed. Each entry is applied to the corresponding index; last entry applies to all remaining indexes. Default: 1. Accepted values: 0, 1 or 2.
`--create-fastq-for-index-reads`	Create FASTQ files also for Index Reads. Generating FASTQ files is based on the following: • The index read masks are specified from the --use-bases-mask option. • The RunInfo.xml file when the --use-bases-mask option is not used.
`--ignore-missing-bcls`	Missing or corrupt BCL files are ignored. Assumes 'N'/'#' for missing calls
`--ignore-missing-filter`	Missing or corrupt filter files are ignored. Assumes Passing Filter for all clusters in tiles where filter files are missing.
`--ignore-missing-positions`	Missing or corrupt positions files are ignored. If corresponding position files are missing, bcl2fastq writes unique coordinate positions in FASTQ header.
`--minimum-trimmed-read-length`	Minimum read length after adapter trimming. bcl2fastq trims the adapter from the read down to the value of this parameter. If there is more adapter match below this value, then those bases are masked, not trimmed (replaced by N rather than removed). Default: 35

Option	Description
--mask-short-adapter-reads	This option applies when a read is shorter than the length specified by --minimum-trimmed-read-length (note that the read does not specifically have to be trimmed for this option to trigger, it need only fall below the –minimum-trimmed-read-length for any reason). These parameters specify the following behavior: If the number of bases left after adapter trimming is less than --minimum-trimmed-read-length, force the read length to be equal to --minimum-trimmed-read-length by masking adapter bases (replace with Ns) that fall below this length. If the number of ACGT bases left after this process falls below --mask-short-adapter-reads, mask all bases, resulting in a read with --minimum-trimmed-read-length number of Ns. In addition, if a read is shorter than--mask-short-adapter-reads for any reason, it will be masked with Ns. Because it applies when a read is shorter than the value of --minimum-trimmed-read-length, it should be set to a value that is less than or equal to this parameter. If it is set to a greater value, it will automatically default to the same value as--minimum-trimmed-read-length. Default: 22
--tiles	The --tiles argument takes a regular expression to select for processing only a subset of the tiles available in the flow cell. Multiple selections can be made by separating the regular expressions with commas. Examples: To select all the tiles ending with 5 in all lanes: --tiles [0-9][0-9][0-9]5 To select tile 2 in lane 1 and all the tiles in the other lanes: --tiles s_1_0002,s_[2-8]
--use-bases-mask	The --use-bases-mask string specifies how to use each cycle. An n means ignore the cycle. A Y (or y) means use the cycle. An I means use the cycle for the Index Read. A number means that the previous character is repeated that many times. An asterisk [*] means that the previous character is repeated until the end of this read or index (length according to the RunInfo.xml). The read masks are separated with commas: , The format for dual indexing is as follows: --use-bases-mask Y*,I*,I*,Y* or variations thereof as specified. You can also specify the --use-bases-mask multiple times for separate lanes, like this way: --use-bases-mask 1:y*,i*,i*,y* --use-bases-mask y*,n*,n*,y* Where the 1: means: Use this setting for lane 1. In this case, the second --use-bases-mask parameter is used for all other lanes. If this option is not specified, the mask is determined from the 'RunInfo.xml file in the run directory. If it cannot do this determination, supply the --use-bases-mask. When the --use-bases-mask option is specified, the number of index cycles and the length of index in the sample sheet should match.
--with-failed-reads	Include all clusters in the output, even clusters that are non-PF. These clusters would have been excluded by default. Note: This option cannot be applied to CBCL data. On RTA 2 systems, clusters that fail filter are no longer read after cycle 25. On systems other than MiSeq and HiSeq 2500, you will get 25 bases, then all Ns.
--write-fastq-reverse-complement	Generate FASTQ files containing reverse complements of actual data.
--no-bgzf-compression	Turn off BGZF compression, and use GZIP for FASTQ files. BGZF compression allows downstream applications to decompress in parallel. This parameter is available in case a consumer of FASTQ data cannot handle all standard GZIP formats.
--fastq-compression-level	Zlib compression level (1–9) used for FASTQ files. Default: 4
--no-lane-splitting	Do not split FASTQ files by lane.
--find-adapters-with-sliding-window	Find adapters with simple sliding window algorithm. Insertions and deletions of bases inside the adapter sequence are not handled.

NOTE

Do not use the `--no-lane-splitting` option if you want to upload the resulting FASTQ files to BaseSpace. The FASTQ files generated from the `--no-lane-splitting` option are not compatible with the BaseSpace file uploader. Files generated without this option (the default setting) are compatible for upload to BaseSpace.

NOTE

FASTQ files containing failed reads cannot be uploaded to BaseSpace.

Table 14 General Options

Option	Description
`-h,` `--help`	Produce help message and exit
`-v,` `--version`	Print program version information
`-l,` `--min-log-level`	Minimum log level Recognized values: NONE, FATAL, ERROR, WARNING, INFO, DEBUG, TRACE Default: `INFO`

BCL Conversion Output Files

The bcl2fastq2 Conversion Software provides the following output files: output directory has the following characteristics:

- FASTQ Files
- InterOp Files
- ConversionStats File
- DemultiplexingStats File
- AdapterTrimming File
- FastqSummary and DemuxSummary
- HTML Reports
- JSON File

FASTQ Files

The bcl2fastq2 Conversion Software converts *.bcl, *.bcl.gz, *.bcl.bgzf, and .cbcl files into FASTQ files, which can be used as input for secondary analysis. When there is no sample sheet, the software generates a `Undetermined_S0` FASTQ file for each lane and read number combination.

FASTQ File Names

FASTQ files are named with the sample name and the sample number. The sample number is a numeric assignment based on the order that the sample is listed for the run. For example:

Data\Intensities\BaseCalls\samplename_S1_L001_R1_001.fastq.gz

- samplename—The sample name listed for the sample. If a sample name is not provided, the file name includes the sample ID.
- S1—The sample number based on the order that samples are listed for the run starting with 1. In this example, S1 indicates that this sample is the first sample listed for the run.

  NOTE

  Reads that cannot be assigned to any sample are written to a FASTQ file for sample number 0, and excluded from downstream analysis.
- L001—The lane number.
- R1—The read. In this example, R1 means Read 1. For a paired-end run, a file from Read 2 includes R2 in the file name. When generated, the Index Reads are I1 or I2.
- 001—The last segment is always 001.

FASTQ files are compressed in the GNU zip format, as indicated by *.gz in the file name. FASTQ files can be uncompressed using tools such as gzip (command-line) or 7-zip (GUI).

FASTQ File Format

FASTQ file is a text-based file format that contains base calls and quality values per read. Each record contains 4 lines:

- The identifier
- The sequence
- A plus sign (+)
- The quality scores in a +33 offset ASCII encoded format

The identifier is formatted as:

@Instrument:RunID:FlowCellID:Lane:Tile:X:Y:UMI ReadNum:FilterFlag:0:IndexSequence or SampleNumber

Example:

```
@SIM:1:FCX:1:2106:15337:1063:GATCTGTACGTC 1:N:0:ATCACG
GATCTGTACGTCTCTGCNTCACCTCCACCGTGCAACTCATCACGCAGCTCATGCCCTTCGGCTGCCTCCTGGACTA
+
CCCCCGGGGGGGGGGGG#:CFFGFGFGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGEGGFGGG
```

Table 15 Identifiers Table

Identifiers	Description
`@`	Each sequence identifier line starts with @.
`instrument`	The instrument ID.
`run number`	The run number on the instrument.
`flowcell ID`	The flowcell ID.
`lane`	The lane number.
`tile`	The tile number.
`x_pos`	The X coordinate of the cluster.
`y_pos`	The Y coordinate of the cluster.
`UMI`	[Optional] The Unique Molecular Identifiers (UMIs) are restricted to A/T/G/C/N. The UMI sequences for Read 1 and Read 2 are separated by a plus sign (+) when the UMIs are specified in the sample sheet.
`read`	**Read 1**—Single read. **Read 2**—Paired-end read.
`is filtered`	**Y**—The read is filtered (only showing when --with-failed-reads option is applied). **N**—The read is not filtered.
`control number`	**0**—No control bits are turned on. **Even number**—Control bits are turned on.
`index sequence or sample number`	The Index reads are restricted to A/T/G/C/N. When an indexed sample sheet is used, the index sequence is written to the end of the read identifier. If an unindexed sample sheet is used (single sample per lane), the sample number is written to the read identifier.

FASTQ Compression

FASTQ files are compressed in the GNU zip format, as indicated by *.gz in the file name. FASTQ files can be uncompressed using tools such as gzip (command-line) or 7-zip (GUI).

The BGZF variant facilitates parallel decompression of the FASTQ files by downstream applications. If a downstream application cannot handle the BGZF variant, it can be turned off with the `--no-bgzf-compression` command line.

Quality Scores

A quality score, or Q-score, is a prediction of the probability of an incorrect base call. A higher Q-score implies that a base call is more reliable.

Based on the Phred scale, the Q-score serves as a compact way to communicate small error probabilities. Given a base call, X, the probability that X is not true, P(~X), results in a quality score, Q(X), according to the relationship:

$$Q(X) = -10 \log_{10}(P(\sim X))$$

where P(~X) is the estimated error probability.

The following table shows the relationship between the quality score and error probability.

Quality Score Q(X)	Error Probability P(~X)
Q40	0.0001 (1 in 10,000)
Q30	0.001 (1 in 1,000)
Q20	0.01 (1 in 100)
Q10	0.1 (1 in 10)

For more information on the Phred quality score, see en.wikipedia.org/wiki/Phred_quality_score.

During the sequencing run, base call quality scores are calculated after cycle 25 and results are recorded in base call (*.bcl) files, which contain the base call and quality score per cycle.

Quality Scores Encoding

In FASTQ files, quality scores are encoded into a compact form, which uses only 1 byte per quality value. In this encoding, the quality score is represented as the character with an ASCII code equal to its value + 33. The following table demonstrates the relationship between the encoding character, its ASCII code, and the quality score represented.

NOTE

When Q-score binning is in use, the subset of Q-scores applied by the bins is displayed.

Table 16 ASCII Characters Encoding Q-scores 0–40

Symbol	ASCII Code	Q-Score
!	33	0
"	34	1
#	35	2
$	36	3
%	37	4
&	38	5
'	39	6
(	40	7
)	41	8
*	42	9
+	43	10
,	44	11
-	45	12
.	46	13
/	47	14
0	48	15
1	49	16
2	50	17
3	51	18
4	52	19
5	53	20

Symbol	ASCII Code	Q-Score
6	54	21
7	55	22
8	56	23
9	57	24
:	58	25
;	59	26
<	60	27
=	61	28
>	62	29
?	63	30
@	64	31
A	65	32
B	66	33
C	67	34
D	68	35
E	69	36
F	70	37
G	71	38
H	72	39
I	73	40

InterOp Files

You can locate the InterOp files in the directory: `<run directory>/InterOp`. The directory contains binary files used by the Sequencing Analysis Viewer (SAV) software to summarize various analysis metrics, such as cluster density, intensities, quality scores, and overall run quality.

The index metrics are stored in the IndexMetricsOut.bin file generated by bcl2fastq2, which has the following binary format:

Byte 0: file version (1)

Bytes (variable length): record:

- 2 bytes: lane number (unint16)
- 2 bytes: tile number (unint16)
- 2 bytes: read number (unint16)
- 2 bytes: number of bytes Y for index name (unint16)
- Y bytes: index name string (string in UTF8Encoding)
- 4 bytes: # clusters identified as index (uint32)
- 2 bytes: number of bytes V for sample name (unint16)
- V bytes: sample name string (string in UTF8Encoding)
- 2 bytes: number of bytes W for sample project (unint16)
- W bytes: sample project string (string in UTF8Encoding)

ConversionStats File

You can locate the ConversionStats.xml file in the directory: `<output directory>/Stats/`, or in the directory specified by the `--stats-dir` option.

The file contains the following information per tile:

- Raw Cluster Count
- Read number
- YieldQ30
- Yield
- QualityScore Sum

The file contains the following information per lane:

- Lane Number

DemultiplexingStats File

You can locate the DemultiplexingStats.xml file in the directory: `<output directory>/Stats/`, or in the directory specified by the `--stats-dir` option. The file contains the following information per lane, barcode, and sample, project.

Also, the file contains the following information for flow cell:

- Barcode Count
- PerfectBarcode Count
- OneMismatchBarcode Count

AdapterTrimming File

The AdapterTrimming file is a text-based file format that contains a statistic summary of adapter trimming for the FASTQ file. You can locate the file in the `<output directory>/Stats/` or in the directory specified by the `--stats-dir` option.

The file contains the following information:

- Lane
- Read

- Project
- Sample ID
- Sample Name
- Sample Number
- TrimmedBases
- PercentageOfBased (being trimmed)

Also, the file contains the fraction of reads with untrimmed bases for each sample, lane, and read number.

FastqSummaryF1L#

The FastqSummaryF1L#.txt file (the # indicates the lane number) contains the number of raw and passed filter reads for each sample number and tile. You can locate the file in the `<output directory>/Stats/` or in the directory specified by the `--stats-dir` option.

DemuxSummaryF1L#

The DemuxSummaryF1L#.txt (the # indicates the lane number) file is only created if the sample sheet contains at least one sample and the sample barcode is provided. This file contains the percentage of each tile that each sample makes up. The file also contains a list of the 1,000 most common unknown barcode sequences, and the total number of reads with each barcode seen (Note: to improve speed, the total for each barcode is estimated using a sampling algorithm, and is approximate).

You can locate the file in the `<output directory>/Stats/` or in the directory specified by the `--stats-dir` option.

HTML Report

The HTML reports are generated from data in the DemultiplexingStats.xml and ConversionStats.xml files. You can locate the reports in the directory: `<output directory>/Reports/html/`, or in the directory specified by the `--reports-dir` option.

The Flowcell Summary contains the following information:

- Clusters (Raw)
- Clusters (PF)
- Yield (MBases)

NOTE

For HiSeq X, HiSeq 4000, and HiSeq 3000, the number of raw clusters is actually the number of wells on the flow cell that could potentially be seeded. The value is the same in all cases.

The Lane Summary provides the following information for each project, sample, and index sequence specified in the sample sheet:

- Lane #
- Clusters (Raw)
- % of the Lane
- % Perfect Barcode
- % One Mismatch
- Clusters (Filtered)

- Yield
- % PF Clusters
- %Q30 Bases
- Mean Quality Score

The Top Unknown Barcodes table in the HTML report provides the count and sequence for the 10 most common unmapped bar codes in each lane.

JSON File

The Java Script Object Notification (JSON) file contains the *.json file extension. The format for the JSON file makes it easier to parse the output data. The data in the JSON file are a combination of all the following files:

- InterOP
- ConversionStats
- DemultiplexingStats
- Adapter Trimming
- FastqSummary and DemuxSummary
- HTML Report
-
- The format of the JSON file is similar to the following example:

```
{
   Flowcell: string //matches Flowcell from RunInfo.xml
   RunNumber: int, //matches Run Number from RunInfo.xml
   RunId: string, //matches Run Id from RunInfo.xml
   ReadInfosForLanes: [ //details per-lane read information
      {
         LaneNumber: int,
         ReadInfos: [
         Number: int, //indicates read 1 or read 2 (possible values: 1 and 2)
         NumCycles: int, //indicates number of cycles for this read
         IsIndexedRead, bool // indicates whether or not this read is an
            index read
         ]
      }
   ],
   ConversionResults:[ //details the conversion/demultiplexing results
      {
         LaneNumber: int,
         TotalClustersRaw: int, //number of raw clusters in this lane (null
            for HiSeq X)
         TotalClustersPf: int //number of clusters passing filter in this
            lane
         Yield: int, //total yield in this lane
         DemuxResults: [ //do not include undetermined reads in this array
            {
               SampleId: string,
```

```
                SampleName: string,
                IndexMetrics: [ //empty array if no indices were used for
                   demultiplexing this sample
                   {
                      IndexSequence: string, //if there are two indices, then
                         concatenate with '+' character (e.g.
                         "ATCGTCG+TGATCTA")
                      MismatchCounts: {
                         0: int, //count of perfectly matching barcodes
                         1: int //count of barcodes with one mismatch
                      }
                   }
                ],
                NumberReads: int, //number of read pairs identified as
                   index/index-pair
                Yield: int, //number of bases after trimming
                ReadMetrics: [
                   {
                      ReadNumber: int,
                      Yield: int,
                      YieldQ30: int,
                      QualityScoreSum: int,
                      TrimmedBases: int
                   }
                ]
             }
          ]
       }
    ],
    UnknownBarcodes: [ //details all the unknown barcodes for a given lane and
       number of times it was encountered
       {
          Lane: int,
          Barcodes: {
             string: int //example: "ATGAAGAT": 5888
          }
       }
    ]
}
```

Troubleshooting

- If the bcl2fastq2 Conversion Software fails to complete a run, it could be missing an input file or have a corrupt file. View the log file for missing or corrupt files. The exact wording of the file status reported varies depending on the nature of the file corruption. If the problem is the BCL file, launch the `--ignore-missing-bcls` option. See BCL Advanced Options.

- If there is a high percentage of reads assigned as undetermined, view the Top Unknown Barcodes table in the HTML report on the index sequence.
- If the bcl2fastq2 Conversion Software has problems processing Small RNA samples, use the `--minimum-trim-read-length 20` and `--mask-short-adapter-reads 20` command line instead of the default settings.

Appendix: Installation Requirements

The bcl2fastq2 Conversion Software requires the following components:

Component	Requirements
Network Infrastructure	1 Gigabit minimum.
Server Infrastructure	Single multiprocessor or multicore computer running Linux.
Analysis Computer	Run software on the Linux operating systems only.
Memory	32 GB RAM.
Software	We recommend the either the CentOS 6 or the RedHat Enterprise Linux 6 platform.  **NOTE** Other Linux distributions may work if the dependencies are met, but are not officially supported for installation. The following software is required: • zlib • librt • libpthread The following software are required to build the bcl2fastq2 Conversion Software : • gcc 4.8.2 or later (with support for C++11) • boost 1.54 • CMake 2.8.9 • zlib • librt • libpthread

Revision History

Part #	Revision	Date	Description of Change
15051736	02	March 2017	• Updated to support bcl2fastq2 v2.19. • Added NovaSeq file structure information. • Added CBCL file format section. • Revised BCL2FASTQ options.
15051736	01	April 2016	• Updated to support bcl2fastq2 v2.18. • Reformatted the User Guide to Illumina style standards. • Added JSON file and input files list for MiniSeq. • Revised BCL2FASTQ options and sample sheet settings.
15051736	G	July 2015	Updated to software requirements, gcc version.
15051736	F	June 2015	Updated to support bcl2fastq2 v2.17.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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

Illumina Customer Support Telephone Numbers

North America 1.800.809.4566	**Germany** 0800.180.8994	**Singapore** 1.800.579.2745
Australia 1.800.775.688	**Hong Kong** 800960230	**Spain** 900.812168
Austria 0800.296575	**Ireland** 1.800.812949	**Sweden** 020790181
Belgium 0800.81102	**Italy** 800.874909	**Switzerland** 0800.563118
China 400.635.9898	**Japan** 0800.111.5011	**Taiwan** 00806651752
Denmark 80882346	**Netherlands** 0800.0223859	**United Kingdom** 0800.917.0041
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France 0800.911850	**Norway** 800.16836	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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