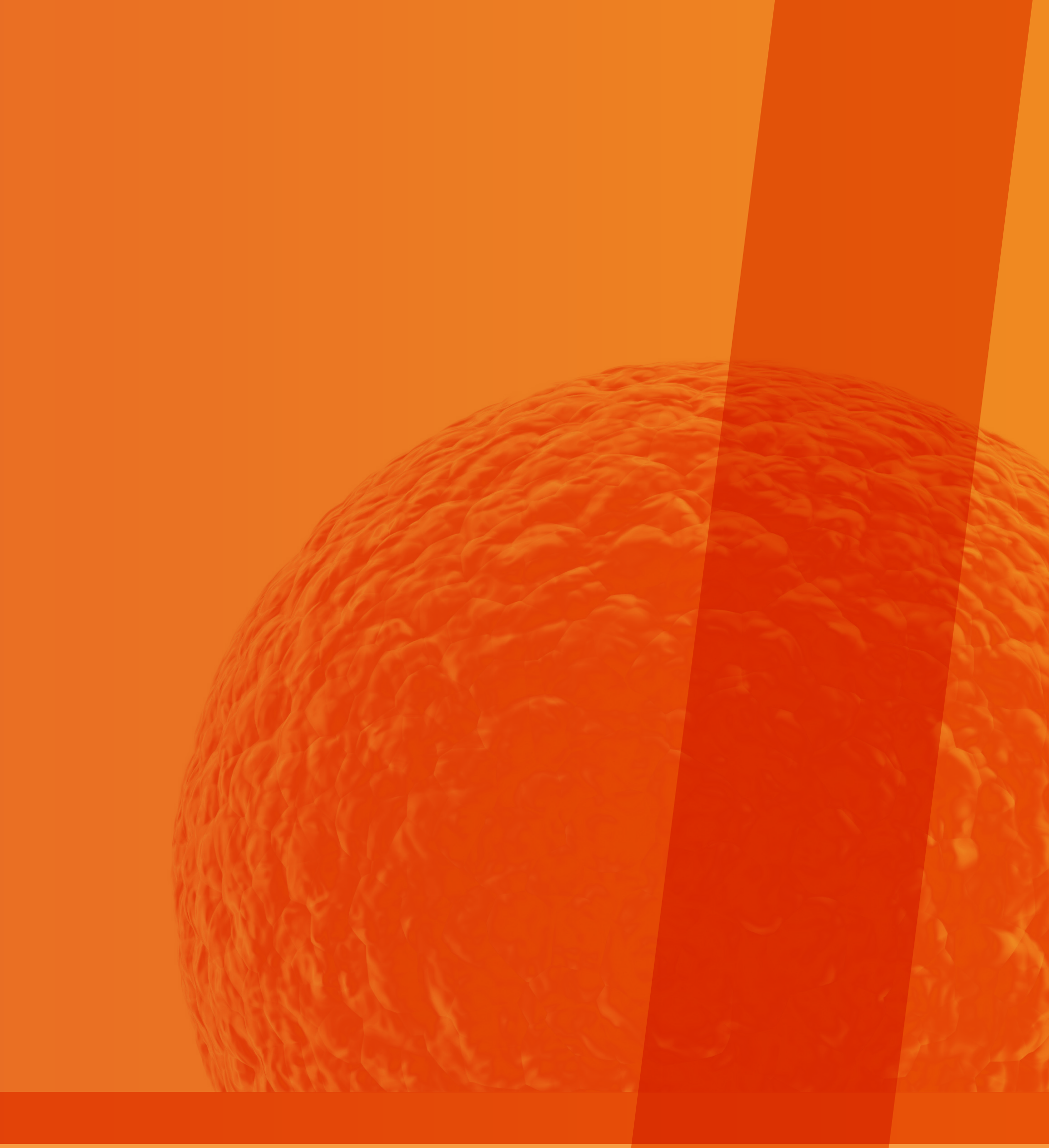

HONEYCOMB™

HIVE™ scRNAseq BeeNet™ v1.1.X Software Guide

Single Cell RNA Sequencing Analysis

v21.10

This product is for research use only.
Not for use in diagnostic procedures.

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BeeNet™ Software Guide

///// Introduction

BeeNet™ is a custom software designed to process data from paired-end Illumina® sequencing of single-cell RNA-seq libraries produced by the HIVE™ scRNAseq Processing Kit. The software consists of a set of programs that receives demultiplexed FASTQ file inputs and yields a transcript and gene count matrix (CM), aligned BAM file, and a quality metrics (QC) file.

This document, along with the accompanying video tutorials **BeeNet™ Download** and **BeeNet™ Running Analysis**, detail how to download, install, and use the software to analyze HIVE™ scRNAseq data. This document is oriented to support a user familiar with Linux™ command line interface.

As part of a single cell RNAseq workflow, BeeNet™ is also hosted on Terra.bio, a cloud-native platform. Terra's graphic user interface (GUI) allows users from any background to run and automate workflows without prior knowledge of command-line tools or cloud computing. The **BeeNetPLUS hosted on Terra.bio v1.0.X Guide** document and **Running Analysis with BeeNetPLUS on Terra.bio** video protocols detail how to use the Terra.bio implementation of BeeNetPLUS.

///// HIVE™ scRNAseq

HIVE™ scRNAseq is a picowell array technology that enables users to collect, store, and process single cells into NGS libraries without specialized instrumentation. HIVE™ scRNAseq libraries are indexed with a sample-specific identifier and Illumina® adapters. Each molecule within a sample is labeled with a unique cell barcode to delineate the cell of origin.

Paired-end sequencing libraries are generated by Illumina® sequencing with Read 1 yielding the individual cell barcode and Read 2 yielding the mRNA sequence.

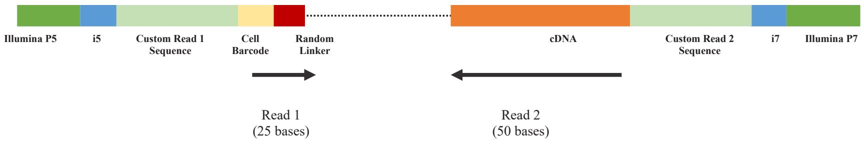


Figure 1: shows the structure of a single library molecule (Illumina P5: Illumina P5 adapter, i5: index 2, i7: index 1, Illumina P7: Illumina P7 adapter)

///// BeeNet™ Workflow

BeeNet™ is an integrated set of programs that will process Read 1 and Read 2 FASTQ files to create Count Matrices as well as aligned BAM and QC files. To run analysis with BeeNet™, you will need a Linux™ system and access to the command-line with read/write/execute permissions.

Setup of BeeNet™ is accomplished through two steps:

1. Download and install the software to your system
2. Download appropriate genome reference files

These steps do not need to be repeated for subsequent analyses on the same system unless a different reference file is required.

Running BeeNet™ to generate count matrices from FASTQ files requires a single command 'analyze' to perform analysis workflow.

BeeNet™ automated analysis follows three main steps:

- QC and pre-processing of raw FASTQ files and cell barcodes
- Alignment and annotation of the reads – outputs a BAM file
- Molecule counting and Count Matrix (CM) creation

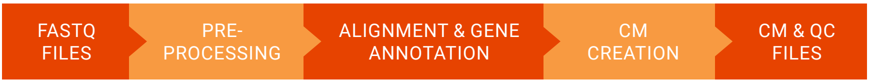


Figure 2: shows the software analysis workflow.

///// System Requirements

BeeNet™ software should be run on Linux™ systems that meet the requirements listed below. The software has been validated using Ubuntu and Debian distributions. Contact your local IT services for instructions on how to set up a Linux™ terminal on a system with the appropriate requirements.
Note: BeeNet should not be run on your personal computer; it should be run on a cloud or cluster-based system with the appropriate requirements:

- RAM: 64GB (note: 128GB is recommended for "mixed-species" genomes)
- Free disk space (after downloading reference files): at least two times the size of your FASTQ files

///// Download and Install BeeNet™

(video protocol – BeeNet™ Download)

Color coding for the rest of the document:

command function flag argument(s)

To download and install the BeeNet™ software, register at **https://download.honeycomb.bio/** by entering your full name, institution, institutional email and accepting the license agreement. You will be emailed a link to download the software, which you can use from a Linux Terminal to install (wget) and give permission to execute commands (chmod).

```
$ wget [your unique link]
```

```
$ chmod +x beenet
```

Additionally, beenet syscheck function can be used to check your system configuration details and available resources for running BeeNet™, to verify system requirements for BeeNet™ are met.

```
$ ./beenet syscheck
```

///// Download Reference

beenet download-ref function can be run without any arguments to view a list of available references. You can download the appropriate reference files based on your sample type. References are large (30 GB+) and will take between 5-15 minutes to download based on your internet speed.

```
$ ./beenet download-ref
```

///// Available Reference Files

Human Genome GRCh38

The reference files were created using the below fasta and gtf:
fasta: http://ftp.ensembl.org/pub/release-104/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz
gtf: http://ftp.ensembl.org/pub/release-104/gtf/homo_sapiens/Homo_sapiens.GRCh38.104.gtf.gz
Source: Ensembl

```
$ ./beenet download-ref 20210603_GRCh38.104
```

Human Genome hg19

The reference files were created using the below fasta and gtf:
fasta: http://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_19/GRCh37.p13.genome.fa.gz
gtf: http://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_19/gencode.v19.annotation.gtf.gz
Source: Gencode

```
$ ./beenet download-ref 20210608_GRCh37.p13
```

Mouse Genome mm10

fasta: http://ftp.ensembl.org/pub/release-104/gtf/mus_musculus/dna_index/Mus_musculus.GRCm39.dna.toplevel.fa.gz
gtf: http://ftp.ensembl.org/pub/release-104/gtf/mus_musculus/Mus_musculus.GRCm39.104.gtf.gz
Source: Ensembl

```
$ ./beenet download-ref  20210714_mm10.104
```

Custom reference files can also be made using the $ ./beenet make-ref function outlined in Advanced Functions section. (p10)

///// BeeNet Analyze™

(Video Protocol -BeeNet™ Running Analysis)

beenet analyze function performs a complete end-to-end analysis of a single sample, incorporating the individual steps of the pipeline into a single workflow. It will process demultiplexed FASTQ files for a single sample and output count matrix and summary files, as well as a BAM file of the aligned and annotated sample data. A typical use of beenet analyze, where the input FASTQ forward (R1) and reverse (R2) pairs are provided as arguments, looks like:

```
$ ./beenet analyze --sample-name=... --ref=... --num-barcodes=# <fq.gz files>
```

beenet analyze flags:

--sample-name=[name of the sample] **(required)**: Used, with a timestamp automatically appended, as a filename prefix for the output files. Only alphanumeric characters, hyphen, period, and underscore are allowed (no leading period or hyphen).

--num-barcodes=[integer]**(required)**: This is the expected number of barcodes in a given sample. Count matrix generation uses this, after sorting by count of reads mapped to genes, as the number of cell barcodes in the sample. Must be an integer. We recommend this number be ~40% of the starting input cell number.

--ref=[absolute path to reference folder] **(required)**: The absolute path to the STAR-indexed genome reference. BeeNet searches the tree provided by the path for the file named genomeParameters.txt and uses this file to determine STAR version compatibility as well as a reference point for the rest of the required STAR index files. If it fin s multiple STAR indices it will report all the ones found.

--FASTQs=[absolute path to text file] As an alternative to passing in long lists of FASTQ file paths on the command line, this parameter specifies a plain text file containing paths to FASTQ files, one pair on each line, R1 and R2 separated by whitespace.

--out=[folder name] Specifies an output directory for output files, this will also contain the temporary STARtmp directory. A timestamp is appended to this directory name so that subsequent runs of BeeNet do not overwrite previous results. If nothing specified, it will output files to working directory.

--mixed=PREFIX1, PREFIX2: Run BeeNet in "mixed genome" mode (differential genome analysis). This will generate separate count matrices and BAM files for the indicated species. *Note: a mixed genome reference files can be used without calling this option but all genes will be outputted in a single CM and BAM file.*

--columns=[cells|genes] This is an optional input for changing the count matrix orientation. If nothing is specified, the default is columns=cells where cells are columns and genes are rows. if --columns=genes specified this will output transposed count matrices where columns are genes and rows are cells.

///// Example Analysis

A test dataset is available for download for users who want to do a test run with BeeNet™. You can download the hosted test data using wget and decompress it using tar

```
$ mkdir FASTQs
```

```
$ chdir FASTQs
```

```
$ wget https://storage.googleapis.com/resources.honeycomb.bio/test-data/HC-TestSample1-FBL.tar.gz
```

```
$ tar -zxvf HC-TestSample1-FBL.tar.gz
```

Example Inputs

- The sample is named "**MySample**": --sample-name=MySample
- There are **5000** cell barcodes in your sample: --num-barcodes=5000
- Using Honeycomb-provided human reference bundle (see: download-refs): --ref=**20210603_GRCh38.104**
- There are 2 lanes worth of paired-end compressed FASTQ files (...fq.gz) for the sample in a directory called **FASTQs/HC-TestSample1-FBL/** put them in the arguments in pairs

To initiate the analysis using the test dataset, you can run the below command:

```
$ ./beenet analyze --sample-name=MySample \
          --num-barcodes=5000 --ref=20210603_GRCh38.104 \
      FASTQs/HC-TestSample1-FBL/HC-TestSample1-FBL_S1_L001_R1.fastq.gz \
      FASTQs/HC-TestSample1-FBL/HC-TestSample1-FBL_S1_L001_R2.fastq.gz \
      FASTQs/HC-TestSample1-FBL/HC-TestSample1-FBL_S1_L002_R1.fastq.gz \
      FASTQs/HC-TestSample1-FBL/HC-TestSample1-FBL_S1_L002_R2.fastq.gz
```

///// BeeNet™ Output File Naming

BeeNet™ outputs an aligned BAM file, which is used to create the count matrices. In addition to the count matrix files there are multiple summary files with QC information related to the sequencing data. Files will be named automatically based in the –sample-name flag as below:

SampleName_DateofAnalysis (YYYYMMDD)_filename.extension

e.g., Sample1_20201201_CM.Reads.tsv.gz

file extensions:

RCM – Read Count Matrix

TCM – Transcript Count Matrix

CMSummary – Count Matrix Summary

///// BeeNet™ Output Files List

Based on your analysis there will either be a single BAM file for that specific species, or if the mixed species analysis was done there will be a BAM file for each species type. The cell barcode for each read is contained in the XC tag for each read.

List of expected output files are as below:

Single species outputs:

Sample1_20201201.bam

Sample1_20201201_RCM.tsv.gz

Sample1_20201201_TCM.tsv.gz

Sample1_20201201_CMSummary.tsv

Sample1_20201201_ ReadsQC.tsv

Sample1_20201201_ SampleQC.tsv

For mixed samples (Human & Mouse):

Sample1_20201201_Human.bam

Sample1_20201201_Mouse.bam

Sample1_20201201_HUMAN_RCM.tsv.gz

Sample1_20201201_HUMAN_TCM.tsv.gz

Sample1_20201201_MOUSE_RCM.tsv.gz

Sample1_20201201_MOUSE_TCM.tsv.gz

Sample1_20201201_CMSummary.tsv

Sample1_20201201_ ReadsQC.tsv

Sample1_20201201_ SampleQC.tsv

///// BeeNet™ Output File Descriptions

All Files with barcodes are alphabetized by the barcodes.

***RCM.tsv.gz**- displays the number of reads for each unique cell barcode that maps to a specific ene in the reference genome

- **Gene:** UniProtKB Gene Name or HGNC Symbol
- **'Headers':** barcodes for each cell - unique cell barcode of 12 bases

***TCM.tsv.gz -** displays the number of unique molecule counts for each transcriptome that mapped to a specific ene in the reference genome

- **Gene:** UniProtKB Gene Name or HGNC Symbol
- **'Headers':** unique transcriptome barcode of 12 bases

***CMSummary.tsv -** displays the number of total Genes and number of molecule counts for each transcriptome barcode

- **Barcode:** unique cell barcode of 12 bases
- **nGenes:** total number of genes for each cell barcode
- **nTran:** total number of molecule counts for each cell barcode

***ReadsQC.tsv -** QC metrics per cell barcode

- **TotalReads:** Total reads for an individual cell barcode
- **MappedReads:** Reads from an individual cell barcode that map to the reference genome
- **ExonReads:** Reads from an individual cell barcode that map to exons
- **FilteredReads:** Total Reads that have passed the filtering prior to alignment
- **PolyAReads:** Reads that contain PolyA
- **5PFReads:** Reads that were filtered out due to having adapter sequence present in 5' end
- **3PFReads:** Reads that were filtered out due to having adapter sequence present in 3' end
- **badBaseBC:** Reads that were filtered out due to 2 or more bases in cell barcode with poor phred scores

***SampleQC.tsv** – QC metrics for all the reads in the FASTQ files for the sample

- **TotalReads:** Total reads in the FASTQ files for the sample
- **MappedReads:** Total Reads that map to the reference genome for the sample
- **ExonReads:** Total Reads that map to exons for the sample
- **FilteredReads:** Total Reads that have passed the filtering prior to alignment for the sample
- **PolyAReads:** Total Reads that were filtered out for containing polyA stretch
- **5PFReads:** Total Reads that were filtered out due to having adapter sequence present in the 5' end
- **3PFReads:** Total Reads that were filtered out due to having adapter sequence present in the 3' end
- **badBaseBC:** Total Reads that were filtered out due to 2 or more bases in cell barcode with poor phred scores

make-ref make custom reference: leverages star to create index files for custom genomes by users and outputs a reference directory with the necessary index files for alignment. Note that creating references requires a minimum of 128GB RAM.

--ref=fasta file of the genome you would like to create indexes for

--gtf=annotation file of the genome you would like to use, please contact support to make sure the gtf file is compatible with the annotation algorithm of Honeycomb

--out=directory where the reference files will be outputted

The individual functions listed below are automatically initiated with the beenet analyze function, but can also be run independently. Please contact support@honeycomb.bio for any questions.

bcin pre-processing of FASTQ files: takes compressed FASTQ files (...fq.gz) as parameters, extracts the cell barcode from the forward read (R1) and encodes it in the name of the reverse read (R2), and writes the single-ended result to stdout. It requires the --sample-name parameter so that it can write out FASTQ pipeline metrics for later use for generating the count matrix.

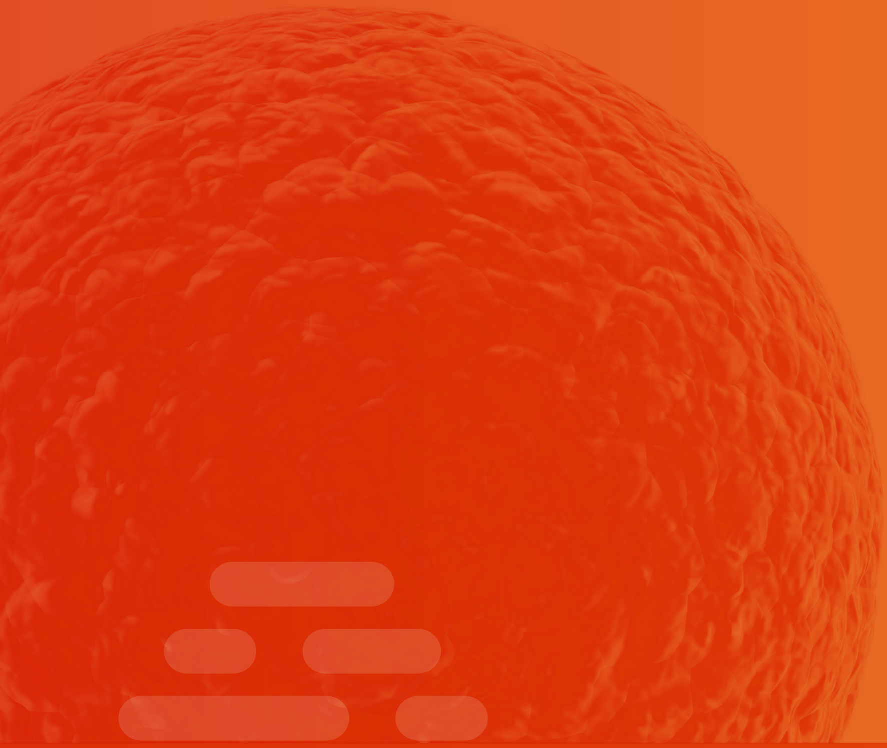
align perform alignment using STAR: calls the embedded STAR aligner with the specified reference, connecting its stdin, stdout, and stderr to BeeNet's stdin, stdout, and stderr. STAR is called with parameters such that it expects single-ended FASTQ on its stdin and produces aligned SAM records on its standard out, and uses STARtmp as a temporary directory.

bcout barcode tagging & alignment filtering: reads SAM records on stdin, filters reads with multiple alignments, decodes cell barcodes from the read name, and writes barcode-tagged SAM records to stdout.

annotate gene annotation: reads SAM records on stdin and annotates them with gene and function according to the provided annotation file (only GTF supported at this time).

count count matrix creation: reads a BAM file, and an optional FASTQ pipeline metrics file, and generates count matrices and summary files.

NOTES



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