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Alignment and feature counting

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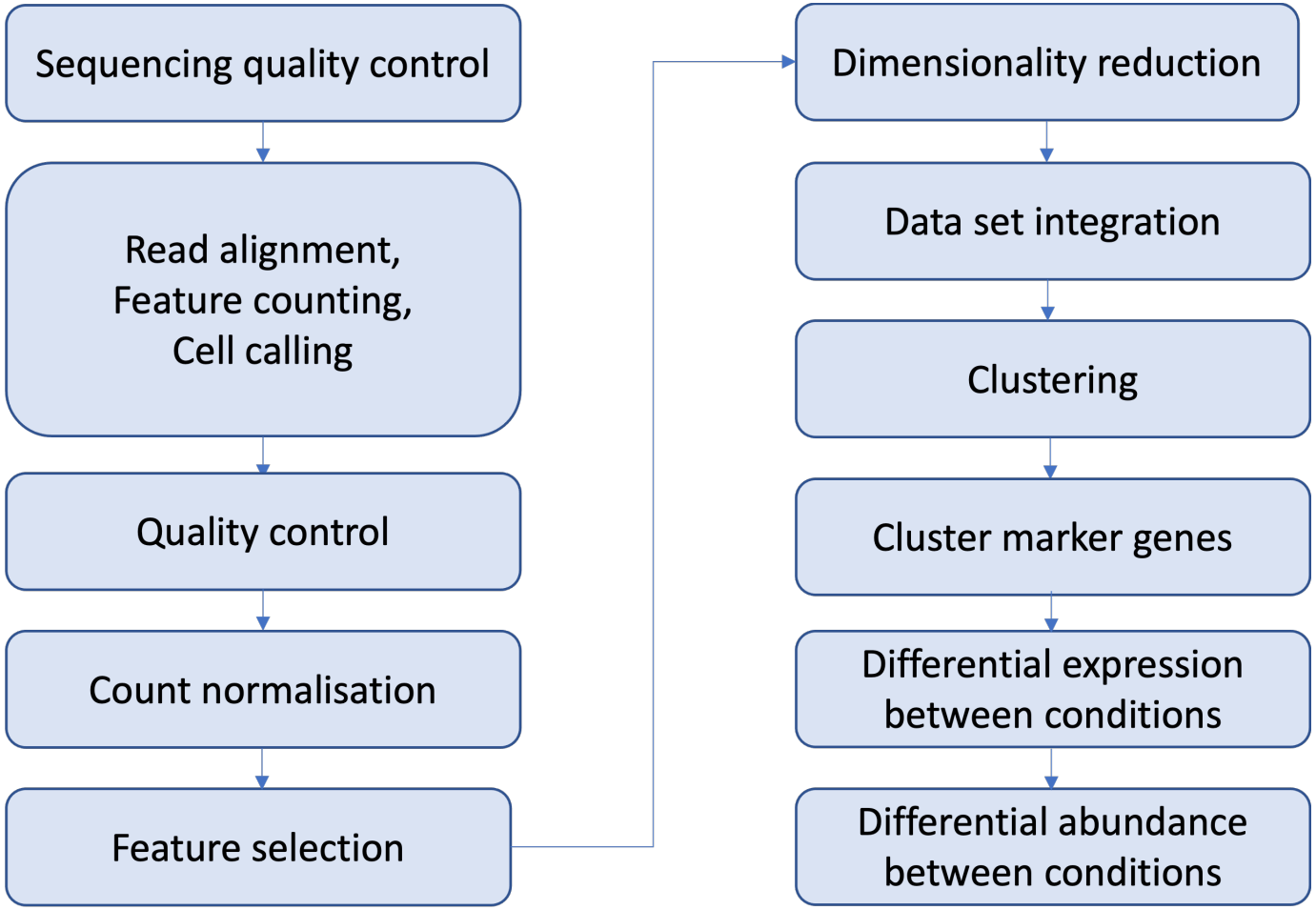
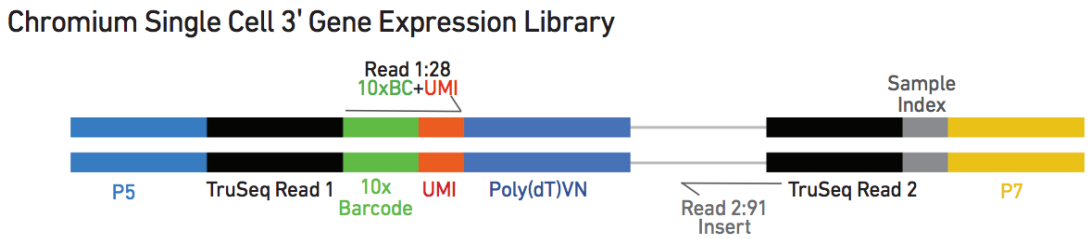
Single Cell RNAseq Analysis Workflow



10x library file structure

The 10x library contains four pieces of information, in the form of DNA sequences, for each “read”.

- **sample index** - identifies the library, with one or two indexes per sample
- **10x barcode** - identifies the droplet in the library
- **UMI** - identifies the transcript molecule within a cell and gene
- **insert** - the transcript molecule



Raw fastq files

The sequences for any given fragment will generally be delivered in 3 or 4 files:

- **I1**: I7 sample index
- **I2**: I5 sample index if present (dual indexing only)
- **R1**: 10x barcode + UMI
- **R2**: insert sequence

QC of Raw Reads - FASTQC

FastQC Report

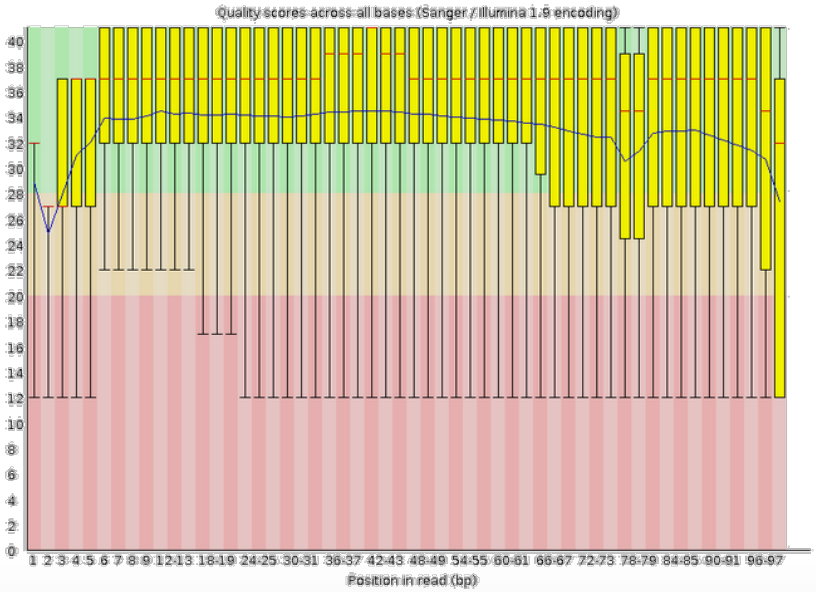
Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content

Basic Statistics

Measure	Value
Filename	SRR9264344_S0_L001_R2_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	330404706
Sequences flagged as poor quality	0
Sequence length	98
%GC	46

Per base sequence quality



QC of Raw Reads - MultiQC

MultiQC v1.11

SLX-21334

- General Stats
- Multi Genome Alignment
  - Summary
  - Lane 2 Statistics
- Barcode Balance
  - Read Counts
  - Barcode Balance
  - Unknown Barcodes
  - Barcode Balance Summary
- FastQC
  - Sequence Counts
  - Sequence Quality Histograms
  - Per Sequence Quality Scores
  - Per Base Sequence Content
  - Per Sequence GC Content
  - Per Base N Content
  - Sequence Length Distribution
  - Sequence Duplication Levels
  - Overrepresented sequences
  - Adapter Content
  - Status Checks
- Single Cell
  - Single Cell Summary
  - Read Mapping
  - Genomic Alignment
  - Barcode Rank & Violin Plots - Lane 2

MultiQC

SLX-21334

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

This report is for the pool SLX-21334 as sequenced in lane 2 of NovaSeq 6000 run 211220_A00489_1183_AHTLCWDRXY.

Report generated on 2021-12-21, 09:12 based on data in: `/mnt/scratcha/sequencing/211220_A00489_1183_AHTLCWDRXY/processing/work/2c/e1a4c6a11d9bf886f7709233aa8114`

Welcome! Not sure where to start? Watch a tutorial video *(6:06)* don't show again ×

Toolbox

General Statistics

Copy table | Configure Columns | Plot | Showing 7/7 rows and 5/7 columns.

Sample Name	M Assigned	M Lost	% Dups	% GC	M Seqs
SLX-21334.HTLCWDRXY.s_2	450.5	25.9			
SLX-21334.HTLCWDRXY.s_2.r_2.lostreads			41.6%	44%	25.9
SLX-21334.SITTA11.HTLCWDRXY.s_2.r_2			59.8%	48%	76.4
SLX-21334.SITTB11.HTLCWDRXY.s_2.r_2			60.7%	47%	80.9
SLX-21334.SITTG10.HTLCWDRXY.s_2.r_2			62.2%	47%	100.4
SLX-21334.SITTH10.HTLCWDRXY.s_2.r_2			63.5%	47%	110.5
SLX-21334.SITTH9.HTLCWDRXY.s_2.r_2			59.9%	47%	82.3

Multi Genome Alignment

MGA (multi-genome alignment) is a quality control tool for high-throughput sequence data developed by the Bioinformatics Core at the Cancer Research UK Cambridge Institute.

Summary

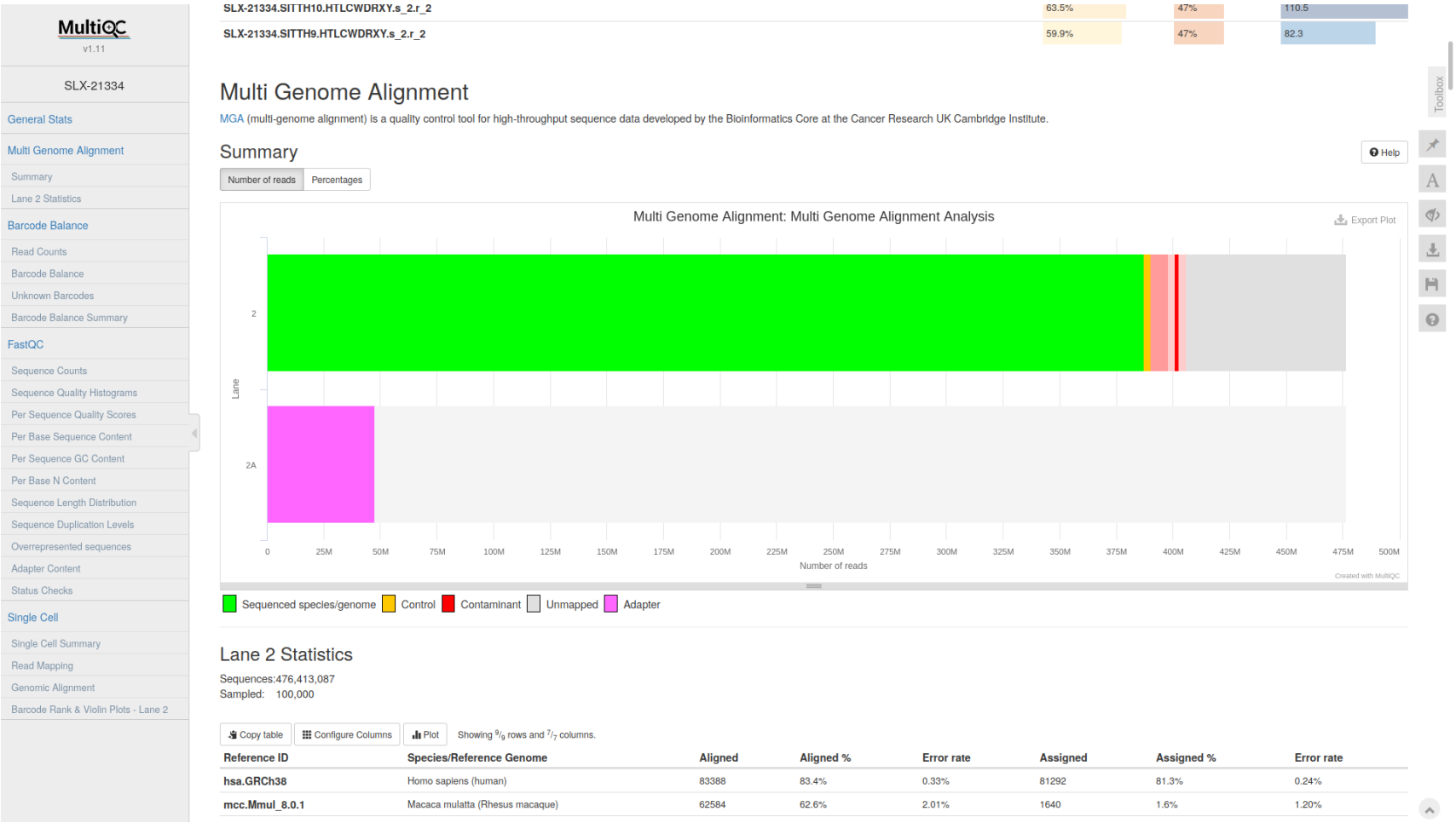
Help

Number of reads | Percentages

Multi Genome Alignment: Multi Genome Alignment Analysis

Export Plot

QC of Raw Reads - MultiQC



QC of Raw Reads - MultiQC

QC of Raw Reads - MultiQC

MultiQC v1.11

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- Single Cell
  - Single Cell Summary
  - Read Mapping
  - Genomic Alignment
  - Barcode Rank & Violin Plots - Lane 2

Single Cell

Single Cell is a plugin to produce reports of single cell CRUK-CI sequencing.

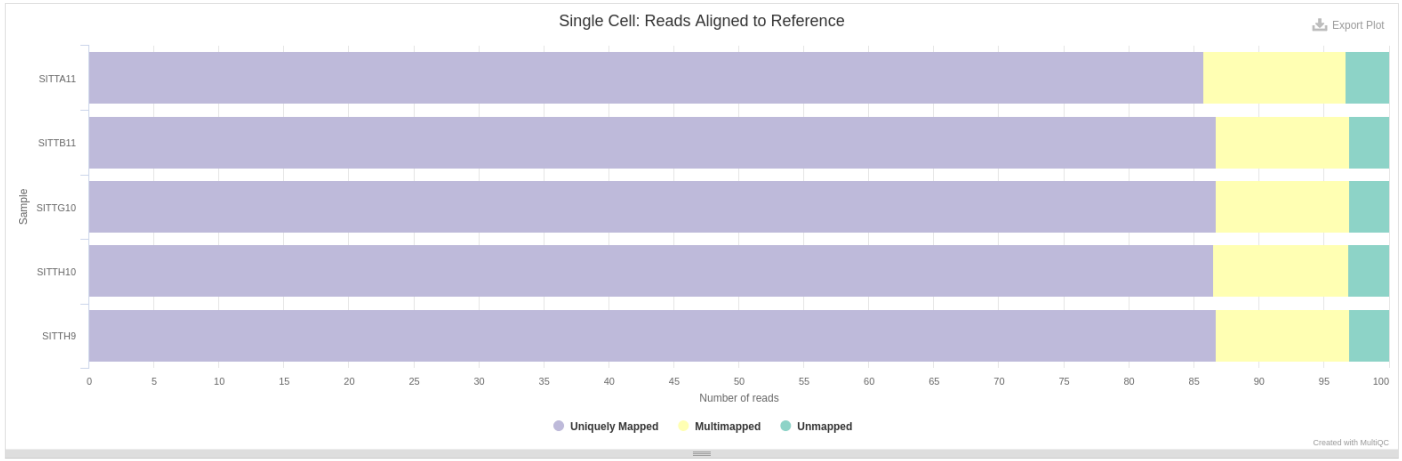
Single Cell Summary

Copy table | Configure Columns | Plot | Showing 5/5 rows and 13/13 columns.

Lane / Barcode	Pool	Sample	Genome	# Cells	% Mapped	Mean R	Median G	# Genes	Median UMI	Mito UMI	# Reads	% Valid	% Saturation
2 / SITTA11	SLX-21334	box12	GRCh38	4 705	78%	16 235	1 813	27 858	5 219	10	76 386 987	97.8%	17.4%
2 / SITTB11	SLX-21334	box13	GRCh38	4 595	79%	17 608	1 939	28 019	5 713	10	80 907 750	97.9%	17.9%
2 / SITTG10	SLX-21334	box15	GRCh38	4 620	82%	21 726	2 250	28 862	7 196	10	100 373 818	97.9%	20.7%
2 / SITTH10	SLX-21334	box16	GRCh38	17 532	82%	6 305	124	29 047	148	2	110 540 893	97.9%	25.3%
2 / SITTH9	SLX-21334	box14	GRCh38	3 970	80%	20 721	2 165	28 130	6 657	10	82 263 870	97.8%	20.0%

Read Mapping

Counts | Percentages



Alignment and counting

The first steps in the analysis of single cell RNAseq data:

- Align reads to genome
- Annotate reads with feature (gene)
- Quantify gene expression

Cell Ranger

- 10x Cell Ranger - This not only carries out the alignment and feature counting, but will also:
  - Call cells
  - Generate a summary report in html format
  - Generate a “cloupe” file

Alternative methods include:

- STAR solo:
  - Generates outputs very similar to CellRanger minus the cloupe file and the QC report
  - Will run with lower memory requirements in a shorter time than Cell Ranger
- Alevin:
  - Based on the popular Salmon tool for bulk RNAseq feature counting
  - Alevin supports both 10x-Chromium and Drop-seq derived data

Obtaining Cell Ranger

What is Cell Ranger? -Soft

https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/lat

10X GENOMICS

Products | Research Areas | Resources | Support | Company

Support › Single Cell Gene Expression › Software

SEARCH | Q&A NEW | CONTACT SUPPORT

SOFTWARE › PIPELINES

CELL RANGER

- Introduction
- Downloads
  - Download Links
  - System Requirements
  - Installing Cell Ranger
  - Release Notes
- Tutorials
  - Getting Started with Cell Ranger
  - Example Data Analysis
  - Build a Custom Reference
  - Design a Custom Panel
- Running Pipelines
  - Computing Options
  - mkfastq
  - Specifying Input FASTQs
  - multi (Cell Multiplexing)
  - count (Gene Expression)
  - count (Targeted GEX)
  - count (Feature Barcode)
  - count (Feature Barcode Only)
  - aggr
  - reanalyze
  - targeted-compare
  - targeted-depth
  - Troubleshooting
- Understanding Outputs

What is Cell Ranger?

Cell Ranger is a set of analysis pipelines that process Chromium single-cell data to align reads, generate feature-barcode matrices, perform clustering and other secondary analysis, and more. Cell Ranger includes four pipelines relevant to the 3' Single Cell Gene Expression Solution and related products:

- **cellranger mkfastq** demultiplexes raw base call (BCL) files generated by Illumina sequencers into FASTQ files. It is a wrapper around Illumina's bcl2fastq, with additional features that are specific to 10x libraries and a simplified sample sheet format.
- **cellranger count** takes FASTQ files from `cellranger mkfastq` and performs alignment, filtering, barcode counting, and UMI counting. It uses the Chromium cellular barcodes to generate feature-barcode matrices, determine clusters, and perform gene expression analysis. The `count` pipeline can take input from multiple sequencing runs on the same GEM well. `cellranger count` also processes Feature Barcode data alongside Gene Expression reads.
- **cellranger aggr** aggregates outputs from multiple runs of `cellranger count`, normalizing those runs to the same sequencing depth and then recomputing the feature-barcode matrices and analysis on the combined data. The `aggr` pipeline can be used to combine data from multiple samples into an experiment-wide feature-barcode matrix and analysis.
- **cellranger reanalyze** takes feature-barcode matrices produced by `cellranger count` or `cellranger aggr` and reruns the dimensionality reduction, clustering, and gene expression algorithms using tunable parameter settings.
- **cellranger multi** is used to analyze Cell Multiplexing data. It inputs FASTQ files from `cellranger mkfastq` and performs alignment, filtering, barcode counting, and UMI counting. It uses the Chromium cellular barcodes to generate feature-barcode matrices, determine clusters, and perform gene expression analysis. The `cellranger multi` pipeline also supports the analysis of Feature Barcode data.

Workflows

Cell Ranger v6.0 (latest)

Cell Ranger tools

Cell Ranger includes a number of different tools for analysing scRNAseq data, including:

- `cellranger mkref` - for making custom references
- `cellranger count` - for aligning reads and generating a count matrix
- `cellranger aggr` - for combining multiple samples and normalising the counts

Preparing the raw fastq files

Cell Ranger requires the fastq file names to follow a convention:

```
<SampleName>_S<SampleNumber>_L00<Lane>_<Read>_001.fastq.gz
```

e.g. for a single sample we may want:

```
SITTA11_S1_L001_I1_001.fastq.gz
SITTA11_S1_L001_I2_001.fastq.gz
SITTA11_S1_L001_R1_001.fastq.gz
SITTA11_S1_L001_R2_001.fastq.gz
```

Unfortunately, the files we receive from the Genomics server will be named like this:

```
SLX-21334.SITTA11.HTLCWDRXY.s_2.i_1.fq.gz
SLX-21334.SITTA11.HTLCWDRXY.s_2.i_2.fq.gz
SLX-21334.SITTA11.HTLCWDRXY.s_2.r_1.fq.gz
SLX-21334.SITTA11.HTLCWDRXY.s_2.r_2.fq.gz
```

Genome/Transcriptome Reference

As with other aligners Cell Ranger requires the information about the genome and transcriptome of interest to be provided in a specific format.

- Obtain from the 10x website for human or mouse (or both - PDX)
- Build a custom reference with `cellranger mkref`

Running `cellranger count`

- Computationally very intensive
- High memory requirements

```
File  Edit  View  Search  Terminal  Help
%h%-$
%h%-$ cellranger count \
>                 --id=SITTA11 \
>                 --fastqs=fastq \
>                 --transcriptome=references/refdata-gex-GRCh38-2020-A \
>                 --sample=SITTA11 \
>                 --localcores=16 \
>                 --localmem=32
```

Cell Ranger outputs

- One directory per sample

```
File  Edit  View  Search  Terminal  Help
%h%-$
%h%-$ ls SITTB11
_cmdline
_filelist
_finalstate
_invocation
_jobmode
_log
_mrosource
outs
_perf
SC_RNA_COUNTER_CS
_sitecheck
SITTB11.mri.tgz
_tags
_timestamp
_uuid
_vdrkill
_versions
%h%-$
```

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Cell Ranger report

10x GENOMICS Cell Ranger • count

SITTA6

Summary | Analysis

14,668
Estimated Number of Cells

20,065
Mean Reads per Cell

1,344
Median Genes per Cell

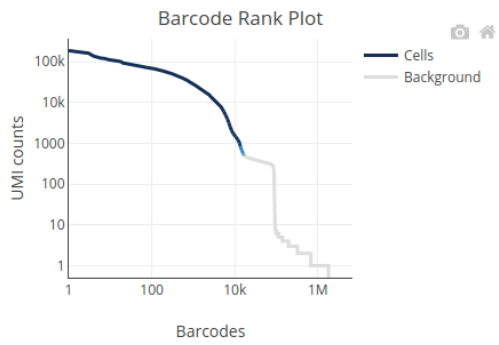
Sequencing

Number of Reads	294,310,066
Number of Short Reads Skipped	0
Valid Barcodes	97.7%
Valid UMIs	100.0%
Sequencing Saturation	18.6%
Q30 Bases in Barcode	96.1%
Q30 Bases in RNA Read	94.6%
Q30 Bases in UMI	95.7%

Mapping

Reads Mapped to Genome	93.6%
Reads Mapped Confidently to Genome	89.7%

Cells



Estimated Number of Cells	14,668
Fraction Reads in Cells	80.8%
Mean Reads per Cell	20,065
Median Genes per Cell	1,344
Total Genes Detected	23,106
Median UMI Counts per Cell	2,928

Sample

Sample ID	SITTA6
Sample Description	

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Loupe Browser

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

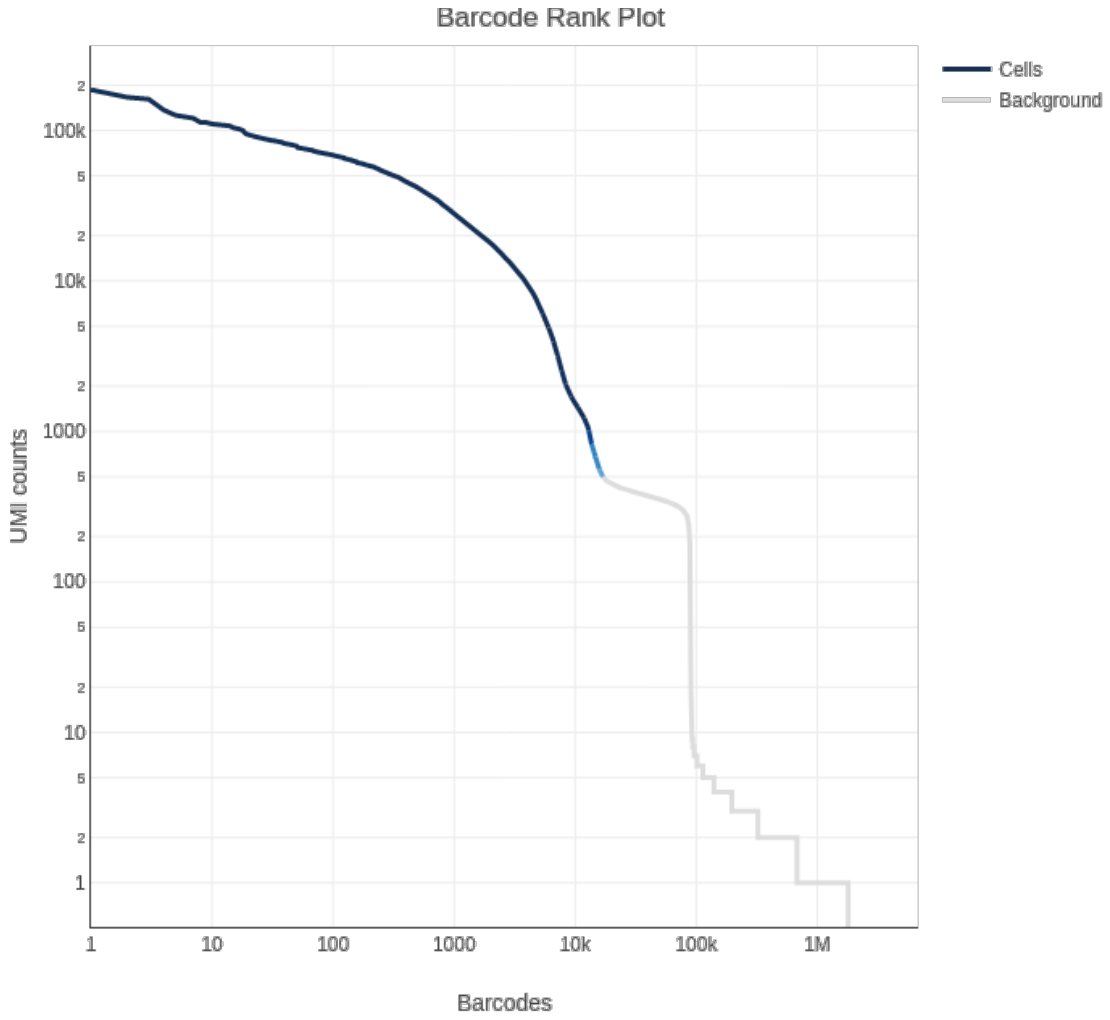
Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
%h%-$ ls SITTB11/outs/raw_feature_bc_matrix
barcodes.tsv.gz
features.tsv.gz
matrix.mtx.gz
%h%-$
```

Cell Ranger outputs

```
File  Edit  View  Search  Terminal  Help
_versions
%h%-$
%h%-$ ls SITTB11/outs
analysis
cloupe.cloupe
filtered_feature_bc_matrix
filtered_feature_bc_matrix.h5
metrics_summary.csv
molecule_info.h5
possorted_genome_bam.bam
possorted_genome_bam.bam.bai
raw_feature_bc_matrix
raw_feature_bc_matrix.h5
web_summary.html
%h%-$
```

Cell Ranger cell calling



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