

Preprocessing (from reads to a count matrix)

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Slides courtesy of:
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Bioinformatician LGTC



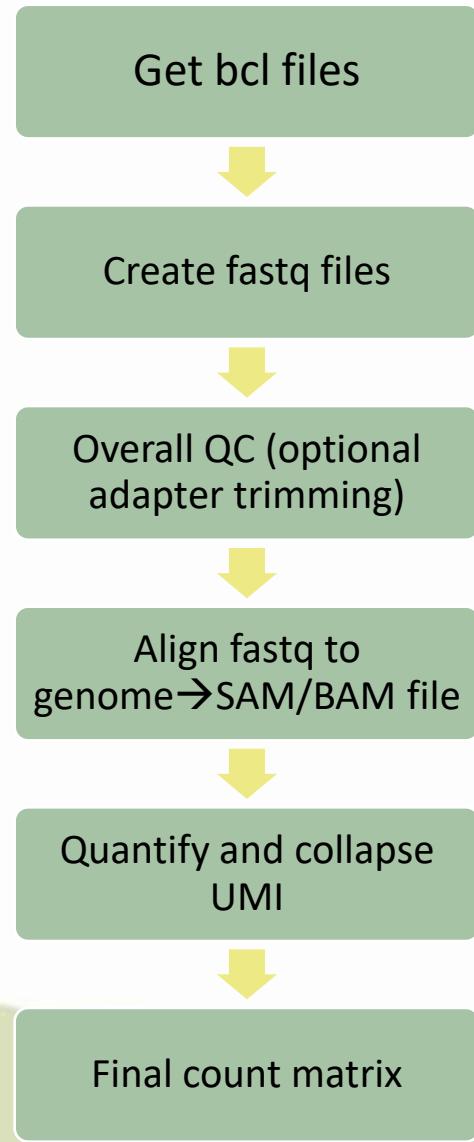
Preprocessing (from reads to a count matrix)

In DNA sequencing, a **read** is an inferred sequence of base pairs corresponding to all or part of a single DNA fragment.

In this lecture

- Sequencing data formats
- Data pre-processing
- 10X Genomic pipeline (Cell Ranger)
- Example outputs

Preprocessing (from reads to a count matrix)



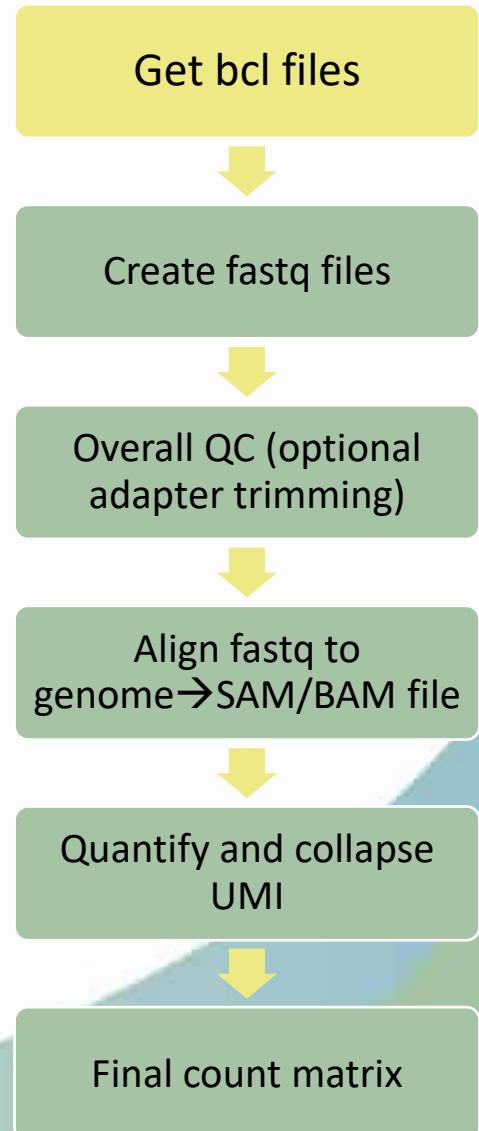
Common file formats in NGS

- bcl
- fastq
- bam
- mtx, tsv
- hdf5 (.h5, .h5ad)

BCL

Raw data files in binary base call format.

Illumina offers **bcl2fastq** Conversion Software to convert BCL files. bcl2fastq is included, standalone conversion software that demultiplexes data and converts BCL files to standard FASTQ file formats for downstream analysis.



Sample demultiplexing

Sequence runs on NGS instruments are typically carried out with multiple samples pooled together. An **index tag** (also called a barcode) consisting of a unique sequence (between 6 and 12bp) is added to each sample so that the sequence reads from different samples can be identified.

The other requirement is a sample sheet

```
[Header],,,,,,,  
IEMFileVersion,4,,  
Date,20-10-2014,,  
Workflow,GenerateFASTQ,,  
Application,FASTQ Only,,  
Assay,Nextera,,  
Description,,  
Chemistry,Amplicon,,  
,,  
[Reads],,,  
151,,  
151,,  
,,  
[Settings],,,  
ReverseComplement,0,,  
Adapter,,  
,,  
[Data],,,  
Lane,Sample_ID,Sample_Name,Sample_Plate,Sample_Well,I7_Index_ID,index,I5_Index_ID,index2  
4,AV_1_HTO,AV_1_HTO,,,ATTACTCG,,  
5,AV_1_HTO,AV_1_HTO,,,ATTACTCG,,
```

```
bcl2fastq --runfolder-dir 190826_E00603_0316_AH3GW3CCX2/ --output-dir HTO/ --sample-sheet samples_HTO.csv --barcode-mismatches 0
```

output-dir: HTO/Reports/html/flowcellID/all/all/all/laneBarcode.html

Flowcell Summary

Clusters (Raw)	Clusters(PF)	Yield (MBases)
1,242,422,208	946,733,762	285,914

Lane Summary

Lane	Project	Sample	Barcode sequence	PF Clusters	% of the lane	% Perfect barcode	% One mismatch barcode	Yield (Mbases)	% PF Clusters	% >= Q30 bases	Mean Quality Score
4	default	AV_1_HTO	ATTACTCG	54,321,382	11.52	100.00		16,405	100.00	46.78	26.39
4	default	Undetermined	unknown	417,222,614	88.48	100.00		126,001	73.60	67.87	31.88
5	default	AV_1_HTO	ATTACTCG	54,933,100	11.56	100.00		16,590	100.00	46.78	26.40
5	default	Undetermined	unknown	420,256,666	88.44	100.00		126,918	74.21	67.77	31.85

Top Unknown Barcodes

Lane	Count	Sequence	Lane	Count	Sequence
4	116,377,900	AGTGGAAC	5	116,758,060	AGTGGAAC
	107,277,740	GTCTCCTT		107,610,520	GTCTCCTT
	79,994,540	TCACATCA		80,406,280	TCACATCA
	74,171,580	CAGATGGG		74,495,820	CAGATGGG
	19,713,380	CAAAAGAT		19,888,220	CAAAAGAT
	705,960	GTCTCCTA		706,360	GTCTCCTA
	629,960	TCACTCAA		638,440	TCACTCAA
	600,500	CAGATGGA		606,040	AGTGAACA
	597,720	AGTGAACA		603,420	CAGATGGA
	564,060	TCCATCAA		574,720	TCCATCAA

FASTQ

- NGS data is often in FASTQ format
 - FASTQ is a text-based format for storing both sequence and its corresponding quality score
 - Four lines per sequence (read)
 - @ followed by the unique sequence identifier
 - The nucleotide sequence
 - + The quality line break
 - The quality scores in ASCII characters

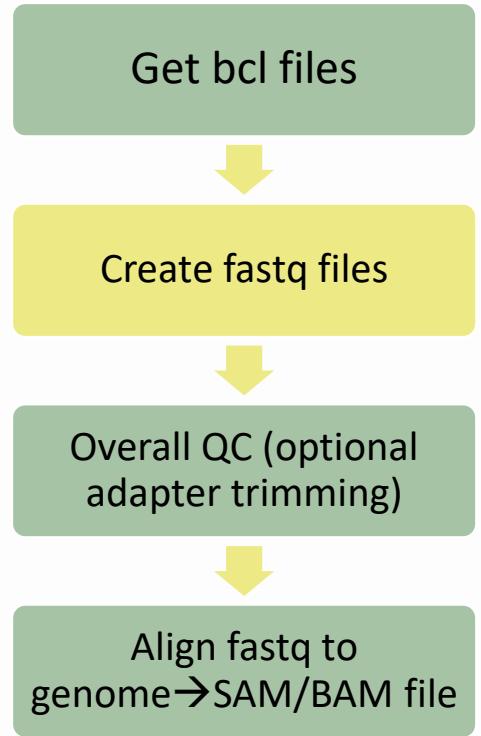
View FASTQ Files

Viewing entire file

```
cat file1.fastq
```

Viewing first 10 lines

head file1.fastq



@A00379:133:HMWGLDSXX:1:1101:1163:1000 2:N:0:GAGGATCT

EE-:::EE-::E-;E-EE-;E;EEEEEE;E;E-;E;E;EEEEEE-;E;:::;EE;E;

QA00379:133:HMWGI_DSXX:1:1101:1253:1000 2:N:0:GAGGATCT

GATGGGTGAAATGCCCTGCTTTAAGTACAAAAGTCGAAAGCTGAGCCACCCAGATTATTTCTGCTGGACCAAGTGTCGCAAAGCTGAACACTGAGACTGAGGCAGATTGAAATGATCCAGG

TCATGCTTAACTCCCTCTTTAACCTACAACTGCAACCACCCAGATTATTCCTCTCTGCAACCTGCAACACTGAACTGACCTGACCCAGATTGAAAATGATCCAGG

FASTQ

Header

@A00379:133:HMWGLDSXX:1:1101:1163:1000 2:N:0:GAGGATCT

Instrument RunID FlowcellID

Read Number
(Paired 2/2)

Index Sequence

+

FF,:::FF,,F,:F,FF,F:FFFF:F:F,,F:F:FFFFF,,:F,,,::FF:F,,,F,FFF:FFFF,,,:,F,,,,:,F,,F:F,F,,,F::F,FFF,,,:,FFFFF,FFFF::,:

Table 1 ASCII Characters Encoding Q-scores 0-40

Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score
!	33	0	/	47	14	=	61	28
"	34	1	0	48	15	>	62	29
#	35	2	1	49	16	?	63	30
\$	36	3	2	50	17	@	64	31
%	37	4	3	51	18	A	65	32
&	38	5	4	52	19	B	66	33
'	39	6	5	53	20	C	67	34
(40	7	6	54	21	D	68	35
)	41	8	7	55	22	E	69	36
*	42	9	8	56	23	F	70	37
+	43	10	9	57	24	G	71	38
,	44	11	:	58	25	H	72	39
-	45	12	;	59	26	I	73	40
.	46	13	<	60	27			

The quality score is associated to a probability of error, of an incorrect base call

Q = -10log₁₀(e) where **e** is the estimated probability of the base call being wrong.

- **Higher Q scores** indicate a smaller probability of error.
- **Lower Q scores** can result in a significant portion of the reads being unusable.

Quality Score

10 (Q10)

20 (Q20)

30 (Q30)

Probability of Incorrect Base Call

1 in 10

1 in 100

1 in 1000

Inferred Base Call Accuracy

90%

99%

99.9%

FASTQC

Before analyzing the data to draw biological conclusions you should always perform some simple quality control

FastQC aims to provide a QC report which can spot problems which originate either in the sequencer or in the starting library material

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
- Kmer Content

Read1

Basic Statistics

Measure	Value
Filename	Pool_1_S1_L001_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	380844038
Sequences flagged as poor quality	0
Sequence length	26
%GC	51

FastQC Report

Summary

- Basic Statistics
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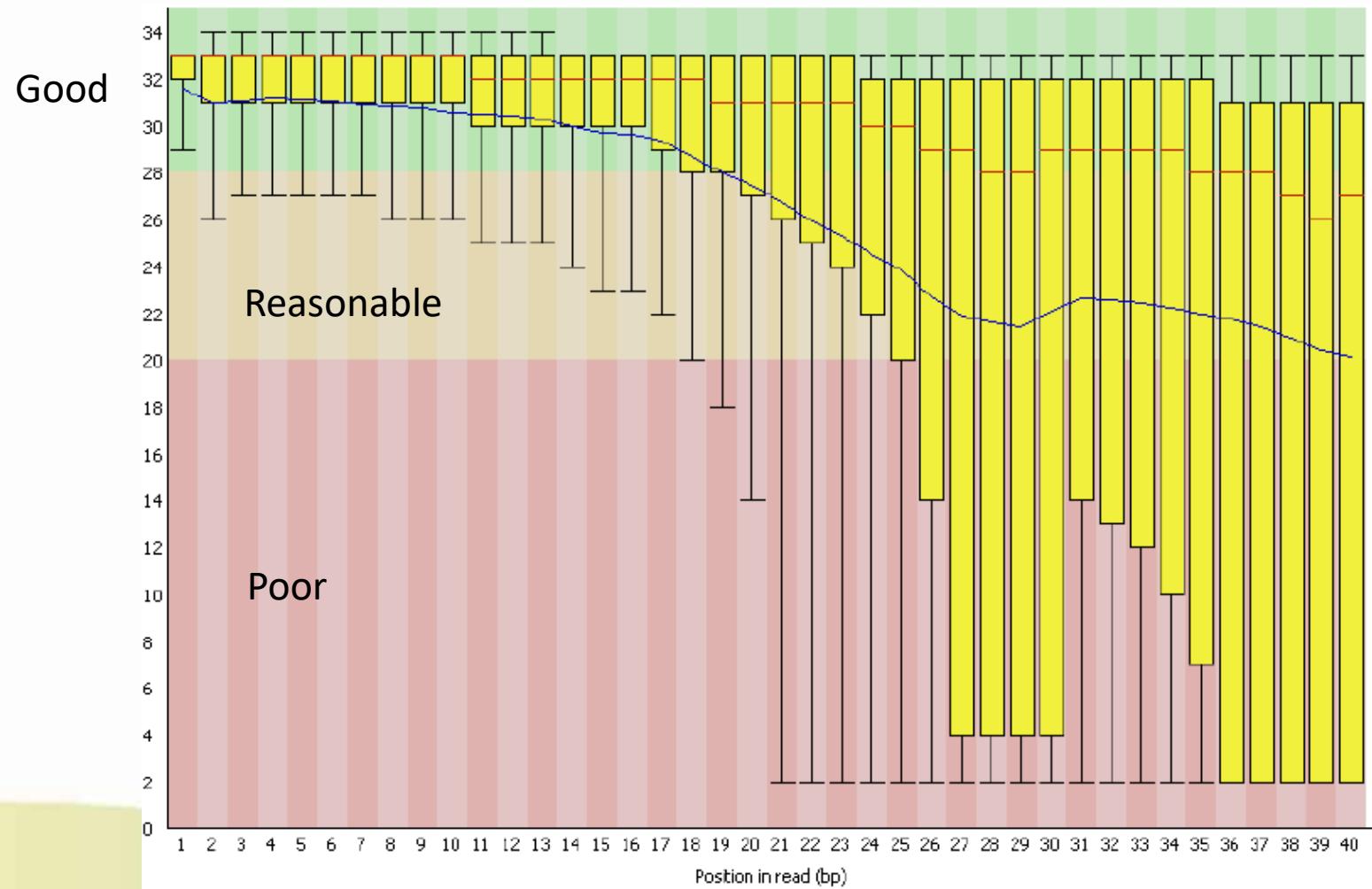
Read2

Basic Statistics

Measure	Value
Filename	Pool_1_S1_L001_R2_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	380844038
Sequences flagged as poor quality	0
Sequence length	150
%GC	36

Per Base Sequence Quality

This view shows an overview of the range of quality values across all bases at each position in the FastQ file.



Overrepresented Sequences

A normal high-throughput library will contain a diverse set of sequences, with no individual sequence making up a tiny fraction of the whole.

This module lists all of the sequence which make up more than 0.1% of the total. Hits must be at least 20bp in length and have no more than 1 mismatch.

Finding a hit doesn't necessarily mean that this is the source of the contamination

Warning

This module will issue a warning if any sequence is found to represent more than 0.1% of the total.

Failure

This module will issue an error if any sequence is found to represent more than 1% of the total.

Typical artifacts

Primers, sequence adapters

✖ Overrepresented sequences

Sequence	Count	Percentage	Possible Source
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	103227946	27.105044506433888	No Hit
GCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	8592717	2.256229884843307	No Hit
AGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	4198600	1.1024460359282295	No Hit
GTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	1116215	0.2930897923102055	No Hit
GG	1018762	0.26750110238039226	No Hit
CAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	1005203	0.26394085234439196	No Hit
AGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	931329	0.24454341070714097	No Hit
GGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTT	563221	0.14788757176238113	No Hit
AAGCAGTGGTATCAACGCAGAGTATTTTTTTTTTTTTT	392982	0.1031871214431352	No Hit

MultiQC

Aggregate results from bioinformatics analyses across many samples into a single report

MultiQC searches a given directory for analysis logs and compiles a HTML report. It's a general use tool, perfect for summarising the output from numerous bioinformatics tools.



Citations 858

[GitHub](#)[Python Package Index](#)[Documentation](#)[92 supported tools](#)[Publication / Citation](#)[Get help on Gitter](#)[Quick Install](#)

```
pip install multiqc    # Install  
multiqc .              # Run  
pip                  conda      manual
```

Need a little more help? See the full installation instructions.

General Statistics

Copy table

Showing 29/29 rows and 3/5 columns.

General Stats
FastQC
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

Sample Name	% Dups	% GC	M Seqs
ARH10_S10_L001_R1_001	69.0%	54%	53.5
ARH10_S10_L001_R2_001	73.0%	53%	53.5
ARH11_S11_L001_R1_001	72.2%	50%	4.8
ARH11_S11_L001_R2_001	73.0%	50%	4.8
ARH12_S12_L001_R1_001	65.9%	57%	26.9
ARH12_S12_L001_R2_001	69.7%	56%	26.9
ARH13_S13_L001_R1_001	50.7%	51%	27.1
ARH13_S13_L001_R2_001	56.6%	51%	27.1
ARH14_S14_L001_R1_001	65.5%	51%	33.2
ARH14_S14_L001_R2_001	68.8%	51%	33.2
ARH15_S15_L001_R1_001	60.4%	47%	22.7
ARH15_S15_L001_R2_001	64.2%	47%	22.7
ARH16_S16_L001_R1_001	65.5%	47%	26.0
ARH16_S16_L001_R2_001	68.9%	47%	26.0
ARH17_S17_L001_R1_001	58.8%	47%	10.0
ARH17_S17_L001_R2_001	61.3%	46%	10.0
ARH18_S18_L001_R1_001	48.2%	50%	28.1

General Stats

FastQC

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

Overrepresented sequences

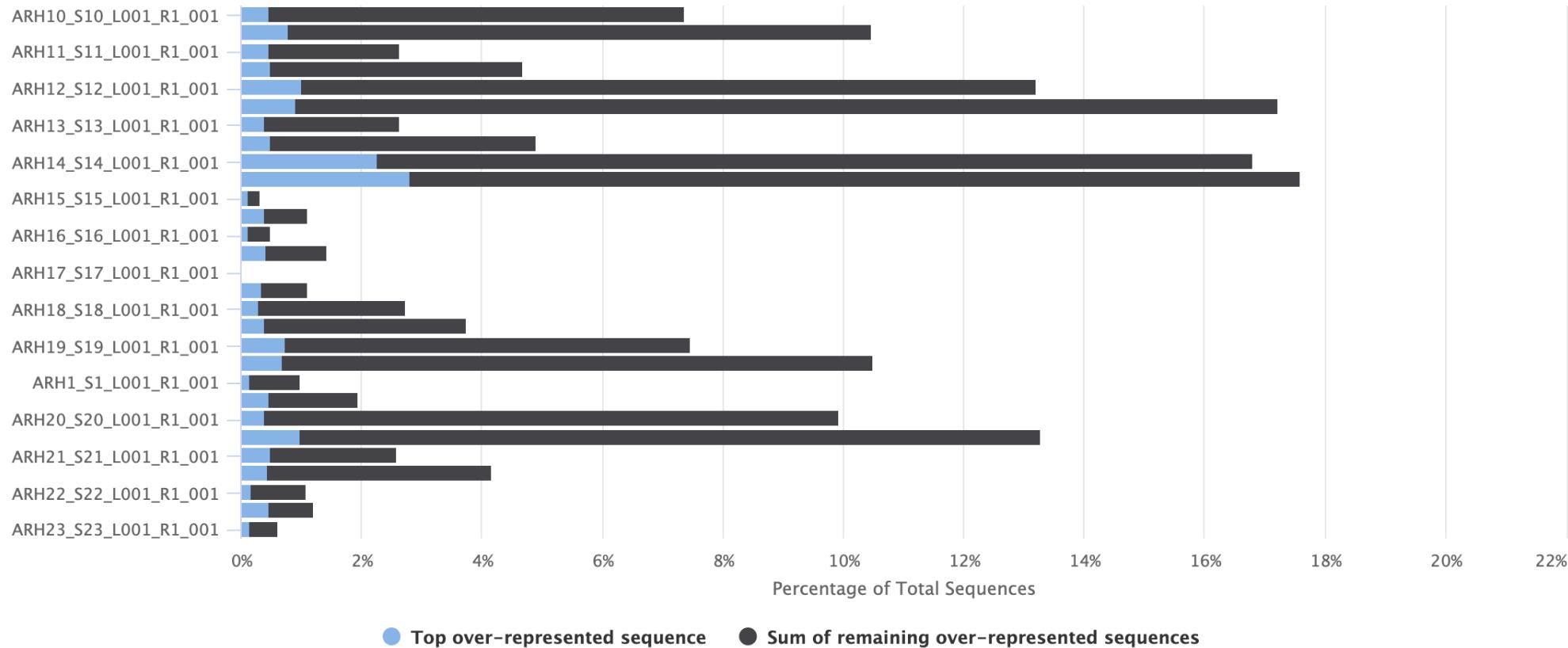
25

3

The total amount of overrepresented sequences found in each library. See the [FastQC help](#) for further information.

Overrepresented sequences

 Export Plot



fastp

<https://github.com/OpenGene/fastp>

fastp

A tool designed to provide fast all-in-one preprocessing for FastQ files. This tool is developed in C++ with multithreading supported to afford high performance.

- [fastp](#)
- [features](#)
- [simple usage](#)
- [examples of report](#)
- [get fastp](#)
 - [install with Bioconda](#)
 - [or download binary \(only for Linux systems, http://opengene.org/fastp/fastp\)](#)
 - [or compile from source](#)
 - [compile from source for windows user with MinGW64-distro](#)
- [input and output](#)
 - [output to STDOUT](#)
 - [input from STDIN](#)
 - [store the unpaired reads for PE data](#)
 - [store the reads that fail the filters](#)
 - [process only part of the data](#)
 - [do not overwrite exiting files](#)
 - [split the output to multiple files for parallel processing](#)
 - [merge PE reads](#)

fastp

UMI example

The original read:

```
@NS500713:64:HFKJJBGXY:1:11101:1675:1101 1:N:0:TATAGCCT+GACCCCCA  
AAAAAAAAGCTACTTGGAGTACCAATAATAAAGTGAGCCCACCTTCCTGGTACCCAGACATTTCAGGAGGTGGGA  
AA + 6AAAAAAEEEEEE/E/EA/E/AEA6EE//AEE66/AAE//EEE/E//E/AA/EEE/A/AEE/EEA//EEEEEEE6EEAA
```

After it's processed with command:

```
fastp -i R1.fq -o out.R1.fq -U --umi_loc=read1 --umi_len=8:
```

```
@NS500713:64:HFKJJBGXY:1:11101:1675:1101:AAAAAAA 1:N:0:TATAGCCT+GACCCCCA  
GCTACTTGGAGTACCAATAATAAAGTGAGCCCACCTTCCTGGTACCCAGACATTTCAGGAGGTGGAAA +  
EEE/E/EA/E/AEA6EE//AEE66/AAE//EEE/E//E/AA/EEE/A/AEE/EEA//EEEEEEE6EEAA
```

Sequence filtering

Adapter trimming
Quality trimming
Trimming fixed length

Tools

Cutadapt
SeqTK
Trimmomatic
fastp

Aligning reads to a reference

FASTQ files contain sequence information that we wish to map to genes in a genome:

1. Select your genome
2. Select your gene annotation file (generally gtf format)
3. Run the alignment program
4. Result of alignment is generally stored in a sam/bam file

Aligners

STAR, Kallisto, Bowtie

Get bcl files



Create fastq files



Overall QC (optional
adapter trimming)



Align fastq to
genome → SAM/BAM file



Quantify and collapse
UMI



Final count matrix

SAM Format

This is the most basic, human readable format, generated by almost every alignment algorithm that exists. It consists of a header, a row for every read in your dataset, and 11 tab-delimited fields describing that read.

SAM Header

The full list of available header fields can be found below

Col	Field	Type	Regexp/Range	Brief description
1	QNAME	String	[!-?A-~]{1,254}	Query template NAME
2	FLAG	Int	[0,2 ¹⁶ -1]	bitwise FLAG
3	RNAME	String	* [!-()+-<>-~] [!-~]*	Reference sequence NAME
4	POS	Int	[0,2 ³¹ -1]	1-based leftmost mapping POSition
5	MAPQ	Int	[0,2 ⁸ -1]	MAPping Quality
6	CIGAR	String	* ([0-9]+[MIDNSHPX=])+	CIGAR string
7	RNEXT	String	* = [!-()+-<>-~] !-~]*	Ref. name of the mate/next read
8	PNEXT	Int	[0,2 ³¹ -1]	Position of the mate/next read
9	TLEN	Int	[-2 ³¹ +1,2 ³¹ -1]	observed Template LENgth
10	SEQ	String	* [A-Za-z.=.]+	segment SEQuence
11	QUAL	String	[!-~]+	ASCII of Phred-scaled base QUALity+33

MapQ (Mapping Quality)

This value reports how well the read aligned to the reference. Different algorithms report it differently but nonetheless, the greater the number the better the alignment (generally).

CIGAR String

This is a shorthand way to encode an entire alignment:

Op	Description
M	Match (alignment column containing two letters). This could contain two different letters (mismatch) or two identical letters. USEARCH generates CIGAR strings containing Ms rather than X's and ='s (see below).
D	Deletion (gap in the target sequence).
I	Insertion (gap in the query sequence).
S	Segment of the query sequence that does not appear in the alignment. This is used with soft clipping, where the full-length query sequence is given (field 10 in the SAM record). In this case, S operations specify segments at the start and/or end of the query that do not appear in a local alignment.
H	Segment of the query sequence that does not appear in the alignment. This is used with hard clipping, where only the aligned segment of the query sequences is given (field 10 in the SAM record). In this case, H operations specify segments at the start and/or end of the query that do not appear in the SAM record.
=	Alignment column containing two identical letters. USEARCH can read CIGAR strings using this operation, but does not generate them.
X	Alignment column containing a mismatch, i.e. two different letters. USEARCH can read CIGAR strings using this operation, but does not generate them.

RefPos: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Reference: C C A T A C T G A A C T G A C T
Read: A C T A G A A T G G G C T G G C T

POS: 5
CIGAR: 3M1I3M1D5M

BAM format

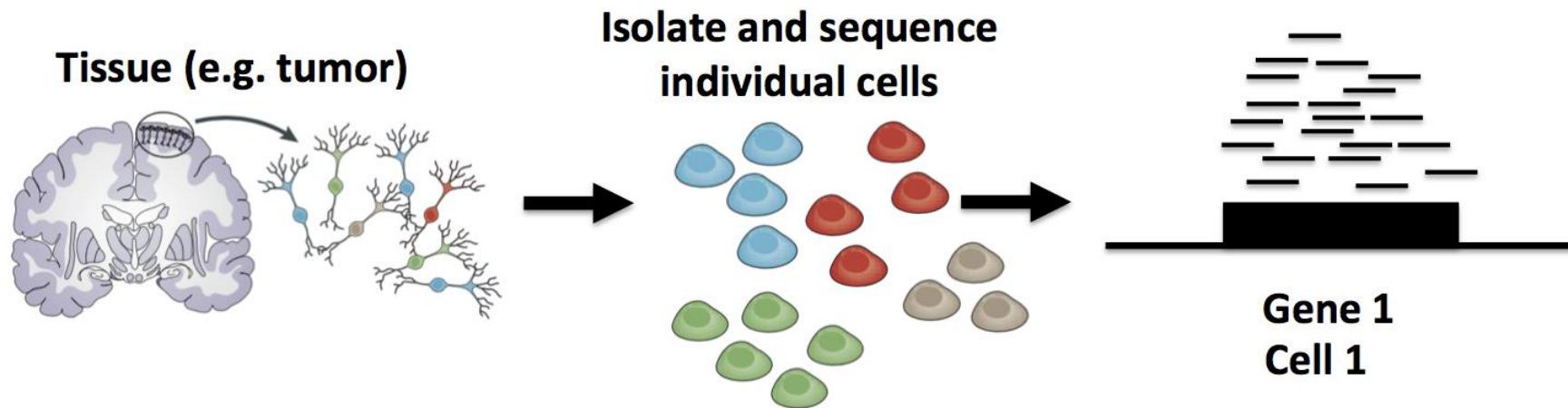
This is the same format except that it is encoded in binary which means that it is significantly smaller than the SAM files and significantly faster to read, though it is not human legible and needs to be converted to another format (i.e. SAM) in order to make sense to us.

Some special tools are needed in order to make sense of BAM, such as [Samtools](#), [Picard Tools](#)

View BAM Files

```
samtools view alignment.bam | head
```

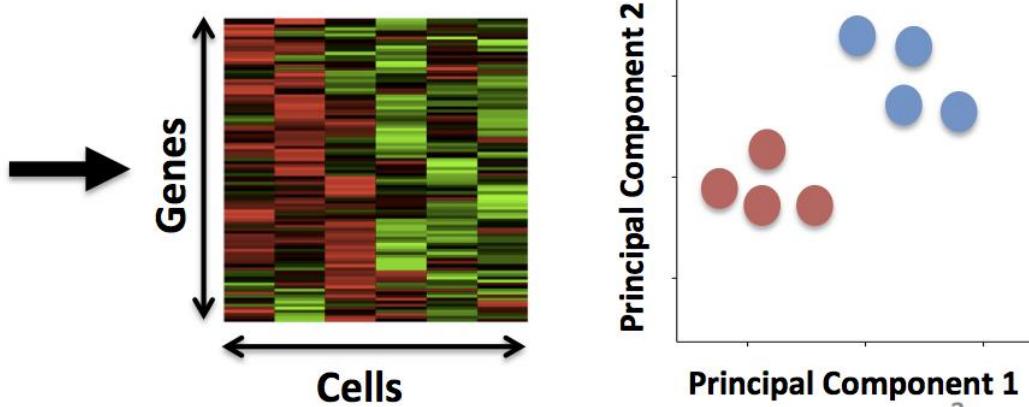
Single-cell RNA-Seq (scRNA-Seq)



Read Counts

	Cell 1	Cell 2	...
Gene 1	18	0	
Gene 2	1010	506	
Gene 3	0	49	
Gene 4	22	0	
...			

Compare gene expression profiles of single cells



Differences between the methods are in how they capture a cell and quantify gene expression (either full-length or tag-based).

- Full-length capture tries to achieve a uniform coverage of each transcript (many reads per transcript).
- Tag-based protocols only capture either the 5'- or 3'-end of each transcript (single read per transcript). Choice in method determines what types of analyses the data can be used for.
- Full-length capture can be used to distinguish different **iso-forms**, where tag-based method is best used for only **gene abundance**.

• **Tag-based 3' counting techniques**

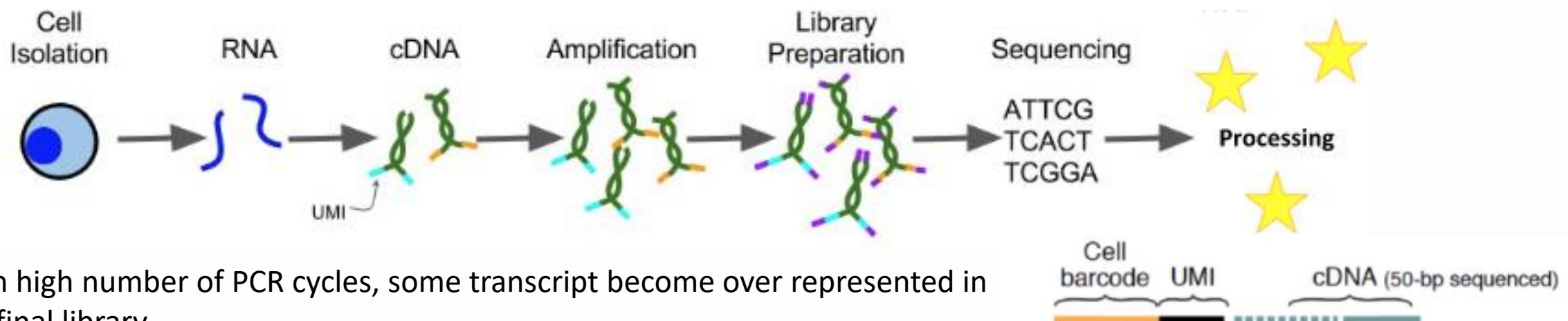
- 1 read per transcript
- Based on polyA
- Expression analysis only
- Fewer reads per cell needed (~20K reads/cell 10x V3+)
- Less noise in expression patterns

• **Full-length**

- Based on polyA
- Expression analysis
- Splicing information
- The more information desired beyond expression, the higher the reads needed per cell (~50K reads/cell to 10M reads/cell)

Challenges in single cell data analysis

Amplification artifacts
Dropouts



With high number of PCR cycles, some transcript become over represented in the final library

UMI are unique molecule identifier added to the transcript during RT step

UMI enable sequencing reads to be assigned to individual transcript molecules and removal of amplification noise and biases

Cell barcode	UMI	cDNA (50-bp sequenced)
AAATTATGACGA	TGTGCTTGGACTGCAC
CCTTAACTGGCA	AGGCCGCGGCTCATAGT
GACCTACGAGTT	AGTTTGTAGCTCATAA
GTAAACGTACG	CTAGCTGTGATTTCT
ACGTACCTTTT	GTGGGGGTATAAGCTC
TTGCCGTGGTGT	TATGGAGGCCAGCACC
AGTCATGTGCGG	CAGGGTTTGTTGGCGT
AAATTATGACGA	AGTTTGTAAGATGGGG
CCAAAGATGTC	TCTAGGCTGGGGACGA
GTAAACGTACG	AAGGCTTGCAAAGTTC
TTTTGACCACTG	CGTGAGGGTTCCAAGG
ACTGTCCATGCCCT	GTTGTATGGTACGT
CCTAAACATAA	ATCCGGTGTTAAACCG

10X technology and Cell Ranger

Cell Ranger is a set of analysis pipelines that process Chromium single-cell RNA-seq output to align reads, generate gene-barcode matrices and perform clustering and gene expression analysis. Cell Ranger includes five pipelines relevant to single-cell gene expression experiments:

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. It can 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.

cellranger reanalyze reruns the dimensionality reduction, clustering, and gene expression algorithms using tunable parameter settings.

cellranger multi is used to analyze Cell Multiplexing data. The cellranger multi pipeline also supports the analysis of Feature Barcode data.

How to run the 10X cellranger software?

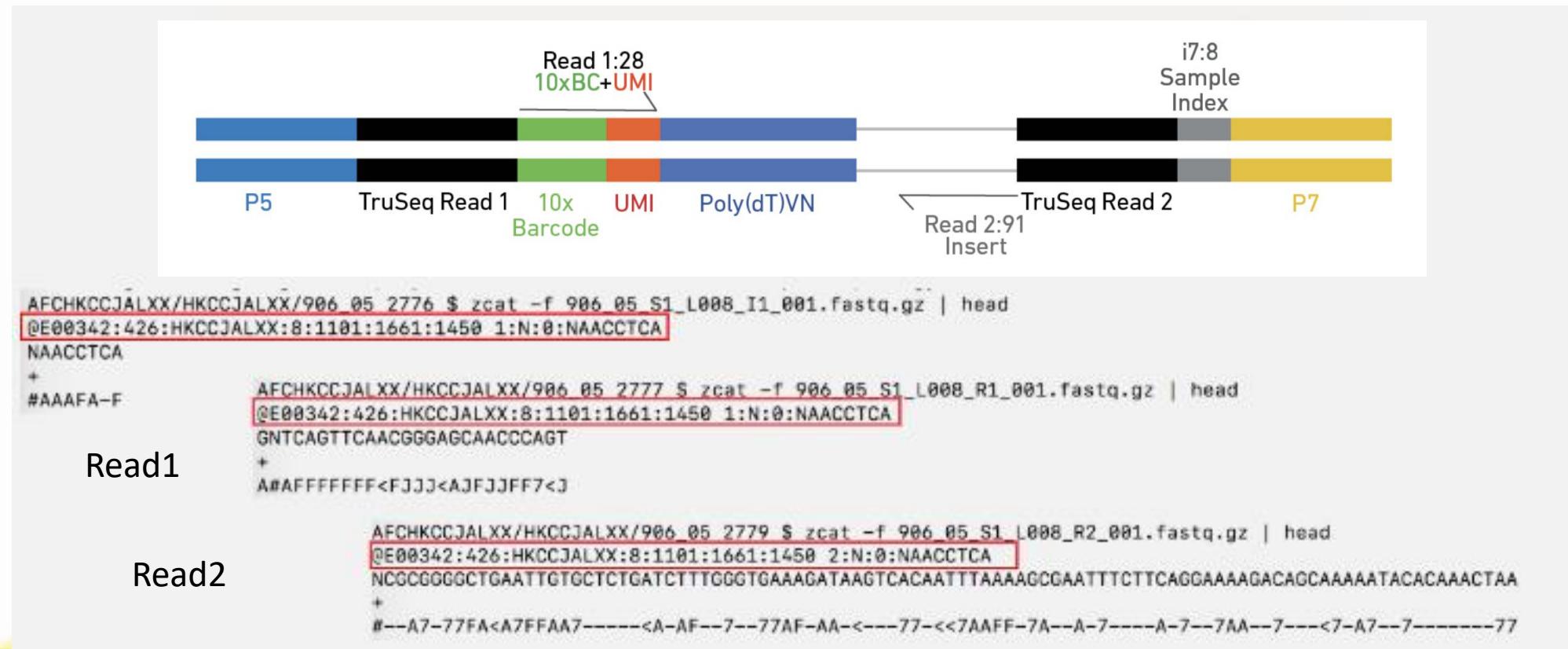
Pipeline download and installation

https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/tutorial_in

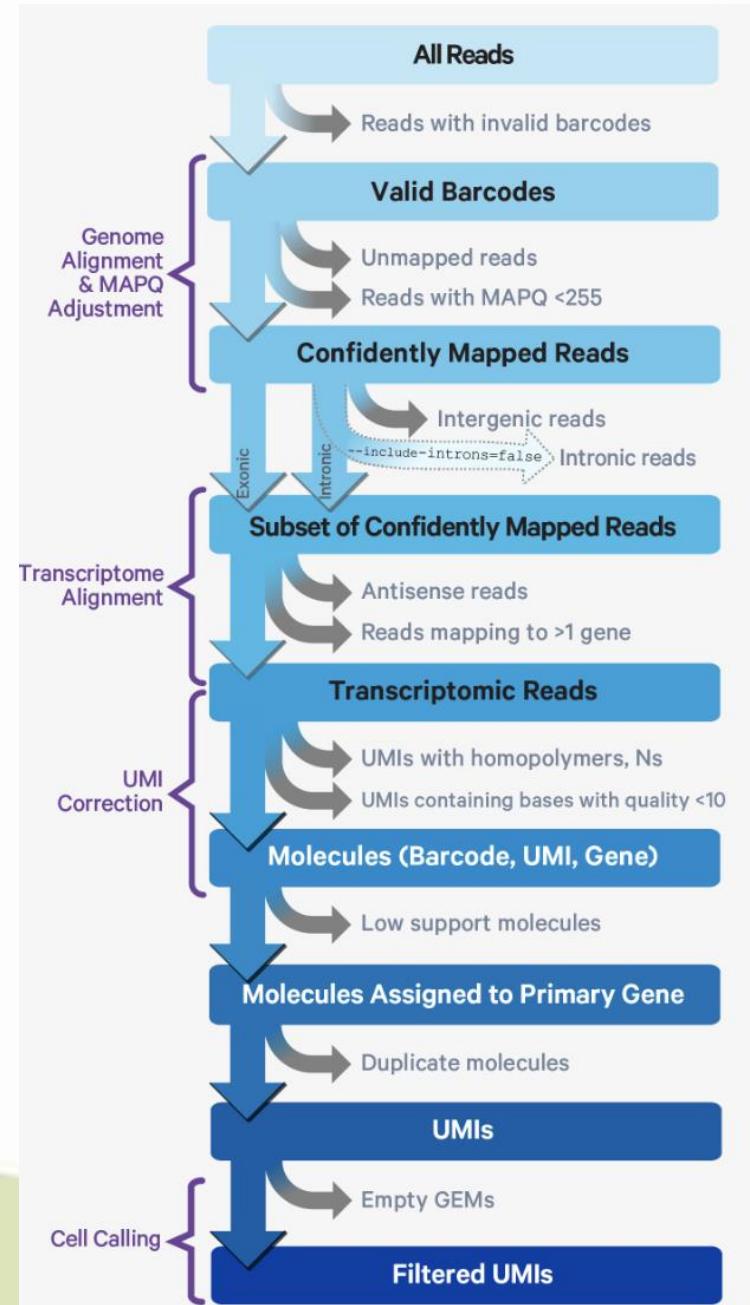
Cell Ranger mkfastq

```
cellranger mkfastq --id=my_data --run=/path/to/bcl --csv=samplesheet.csv
```

Cellranger mkfastq produces :
Index.fastq; R1.fastq; R2.fastq



10X Cell Ranger count pipeline



Gene Expression Algorithms Overview

Read trimming: Cellranger only performs read trimming to 3' gene expression assays.

A full length cDNA construct is flanked by the 30 bp template switch oligo (TSO) sequence on the 5' end and poly-A on the 3' end.

Reads derived from short RNA molecules are more likely to contain either or both TSO and poly-A sequence than longer RNA molecules.

In order to improve mapping, the TSO sequence is trimmed from the 5' end of read 2 and poly-A is trimmed from the 3' end prior to alignment.

Genome Alignment

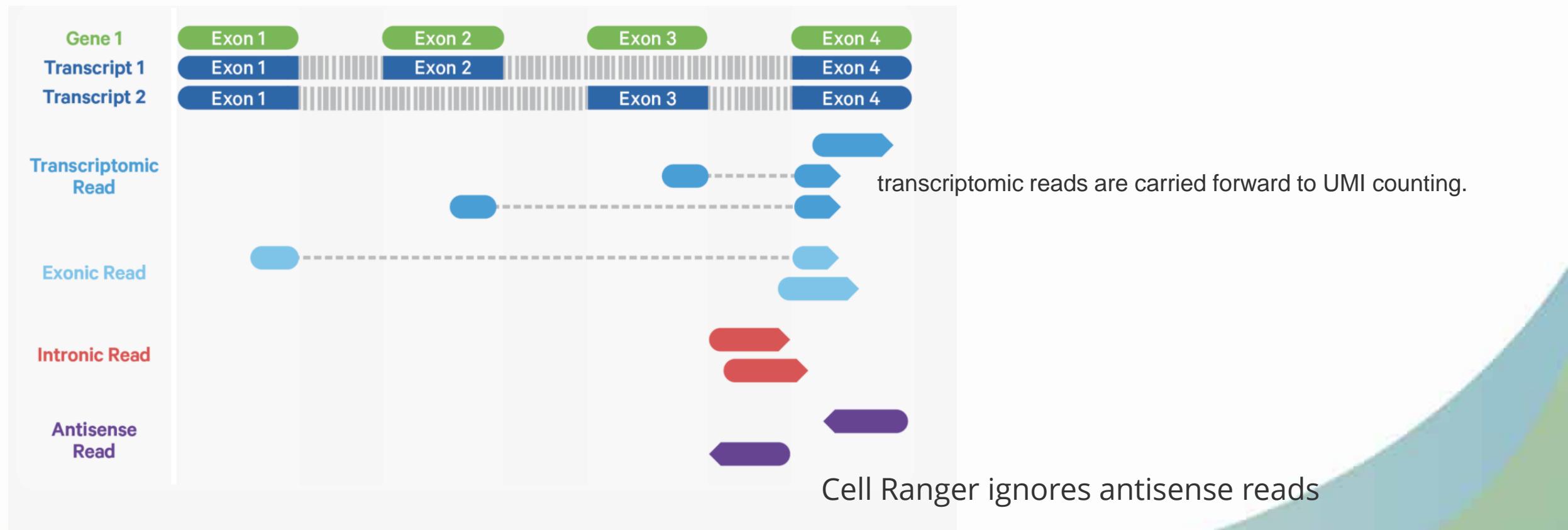
Cell Ranger uses an aligner called [STAR](#), which performs splicing-aware alignment of reads to the genome. Cell Ranger then uses the transcript annotation GTF to categorize the reads into **exonic**, **intronic**, and **intergenic**, and by whether the reads align (confidently) to the genome. A read is exonic if at least 50% of it intersects an exon, intronic if it is non-exonic and intersects an intron, and intergenic otherwise.

MAPQ adjustment

For reads that align to a single exonic locus but also align to 1 or more non-exonic loci, the exonic locus is prioritized and the read is considered to be confidently mapped to the exonic locus with MAPQ 255.

Transcriptome Alignment

Cell Ranger further aligns confidently mapped exonic and intronic reads to annotated transcripts by examining their compatibility with the transcriptome. As shown below, reads are classified based on whether they are sense or antisense and whether they are exonic, intronic, or have a splicing pattern compatible with transcript annotations associated with that gene. Cell Ranger prefers alignments with sense over antisense reads. A read is classified as antisense (purple) if it has any alignments to a transcript exon on the opposite strand and no sense alignments



Starting in **Cell Ranger 7.0**, by default, the cellranger count and cellranger multi pipelines **will include intronic reads** for whole transcriptome gene expression analysis. Any reads that map in the sense orientation to a single gene - the reads labeled transcriptomic (blue) - are carried forward to UMI counting.

The default setting that includes intronic reads is recommended to maximize sensitivity, for example in [cases](#) where the input to the assay consists of nuclei, as there may be high levels of intronic reads generated by unspliced transcripts.

The fraction of gDNA was a small percentage of total UMIs (0.08%) and was a consistent 7-8% of features regardless of transcript length for the PBMC dataset (10X)

Gene Expression Algorithms Overview

Cell barcode and UMI filtering

- **Cell barcodes**

- Must be on static list of known cell barcode sequences
- May be 1 mismatch away from the list if the mismatch occurs at a low-quality position (the barcode is then corrected).

- **UMIs (Unique Molecular Index)**

- Must not be a homopolymer, e.g. AAAAAAAA
- Must not contain N
- Must not contain bases with base quality < 10
- UMIs that are 1 mismatch away from a higher-count UMI are corrected to that UMI if they share a cell barcode and gene.

Filtering cells (Cell Ranger)

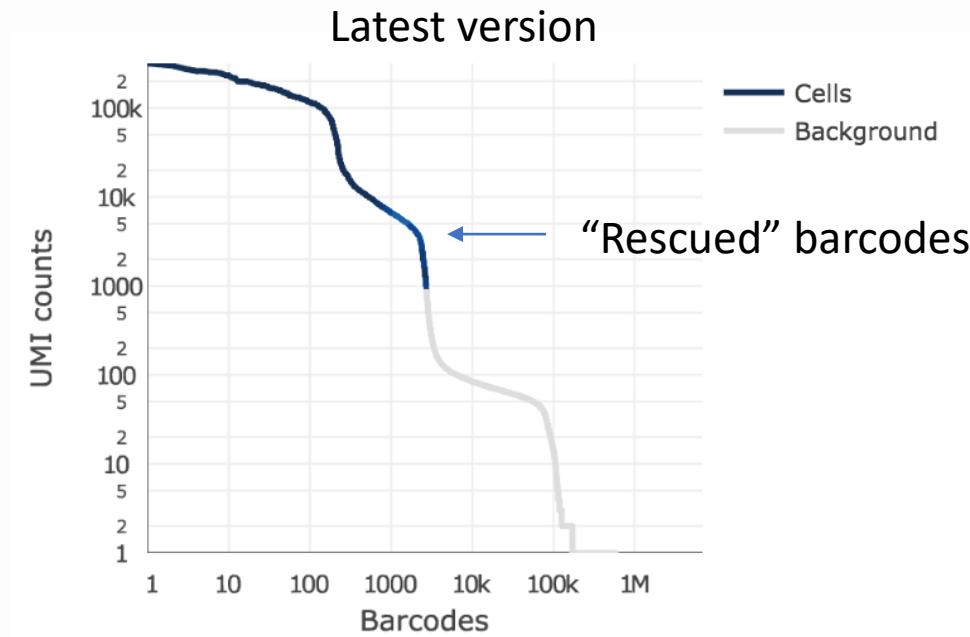
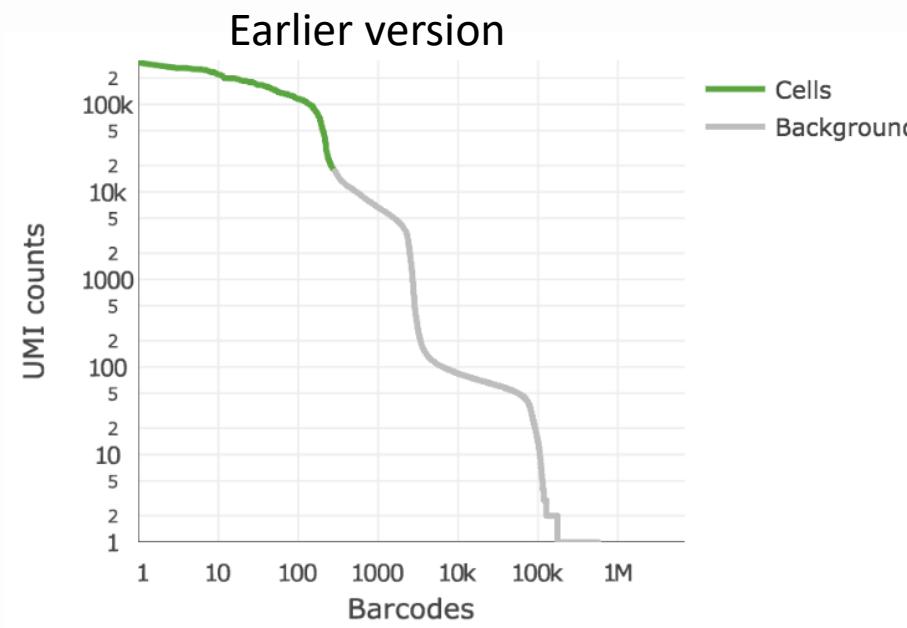
Cellranger 3.0 introduces an improved cell-calling algorithm to identify populations of low RNA content cells, especially when low RNA content cells are mixed into a population of high RNA content cells.
E.g tumor samples often contain large tumor cells mixed with smaller tumor infiltrating lymphocytes (TIL).
The new algorithm is based on the **EmptyDrops** method (Lun et al., 2018).

The algorithm has two key steps:

1. It uses a cutoff based on total UMI counts of each barcode to identify cells. This step identifies the primary mode of high RNA content cells.
2. Then the algorithm uses the RNA profile of each remaining barcode to determine if it is an “empty” or a cell containing partition. This second step captures low RNA content cells whose total UMI counts may be similar to empty GEMs.

Barcodes selection steps

1. The original cellranger cell calling algorithm is used to identify high RNA content cells, using a cutoff based on the total UMI count for each barcode.
2. In the second step, a set of barcodes with low UMI counts that likely represent ‘empty’ GEM partitions is selected. A model of the RNA profile of selected barcodes is created. This second step identifies cells that are clearly distinguishable from the profile of empty GEMs, even though they may have much lower RNA content than the largest cells in the experiment.



Alternative pipelines

10X data or other scRNAseq technologies can also rely on independent pipelines

STARsolo

Alevin

dropEst

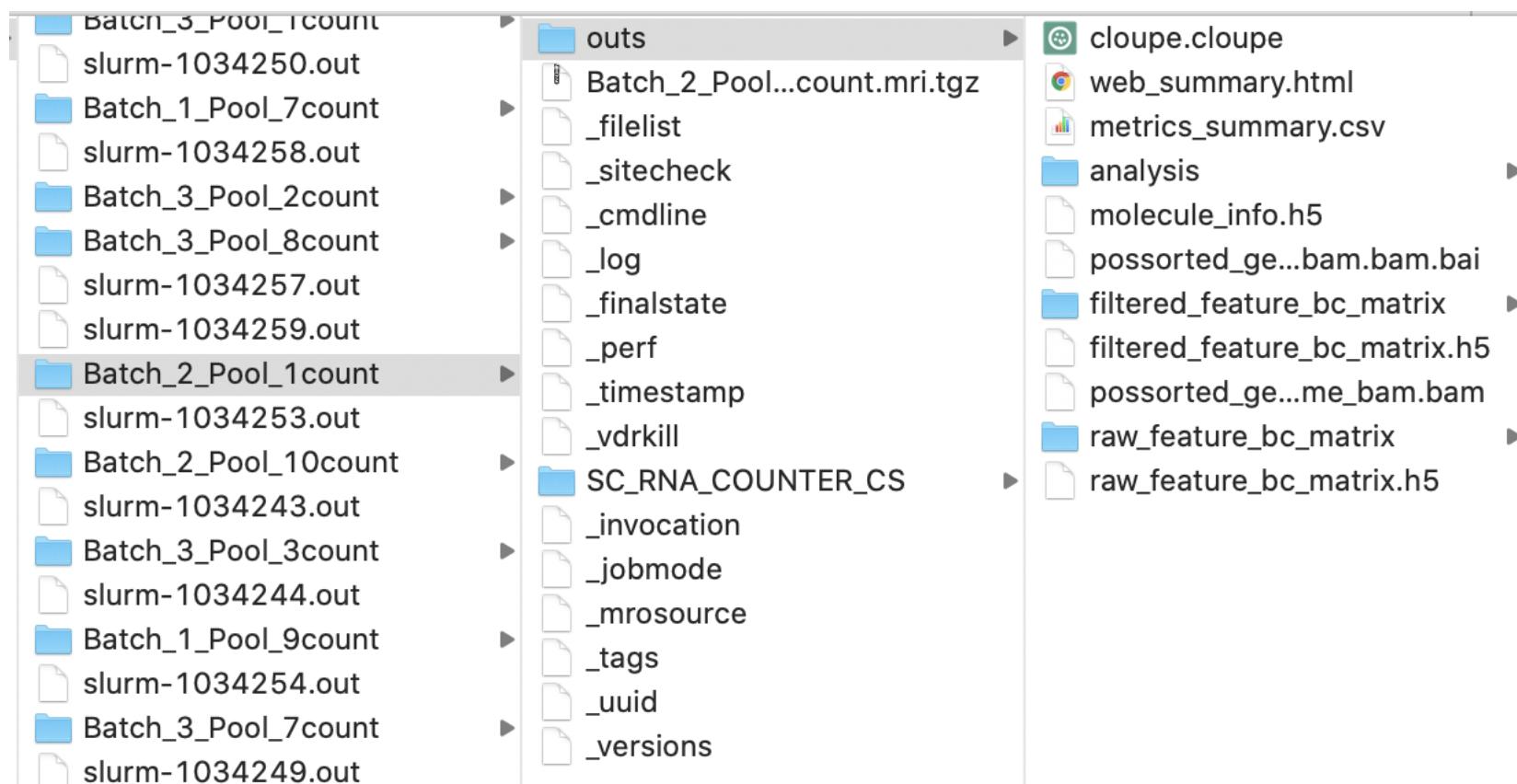
How to run cell ranger count Command-Line Argument Reference

Argument	Description
--id	A unique run ID string: e.g. sample345
--fastqs	Path of the fastq_path folder generated by cellranger mkfastq
--libraries	Path to a libraries.csv file declaring FASTQ paths and library types of input libraries. Required for feature-barcoding analysis. See Feature Barcoding Analysis page for details. When using this argument, --fastqs and --sample must not be passed.
--sample	Sample name as specified in the sample sheet supplied to cellranger mkfastq.
--transcriptome	Path to the Cell Ranger compatible transcriptome reference
--feature-ref	Path to a Feature Reference CSV file declaring the Feature Barcoding reagents in use in the experiment.
--expect-cells	(optional) Expected number of recovered cells.
--force-cells	(optional) Force pipeline to use this number of cells, bypassing the cell detection algorithm.
--chemistry	(optional)
--r1-length	(optional)
--r2-length	(optional) Hard-trim the input R2 sequence to this length.
--include-introns	(optional) Add this flag to count reads mapping to intronic regions. This may improve sensitivity for samples with a significant amount of pre-mRNA molecules, such as nuclei. This flag should be used instead of the deprecated pre-mRNA reference

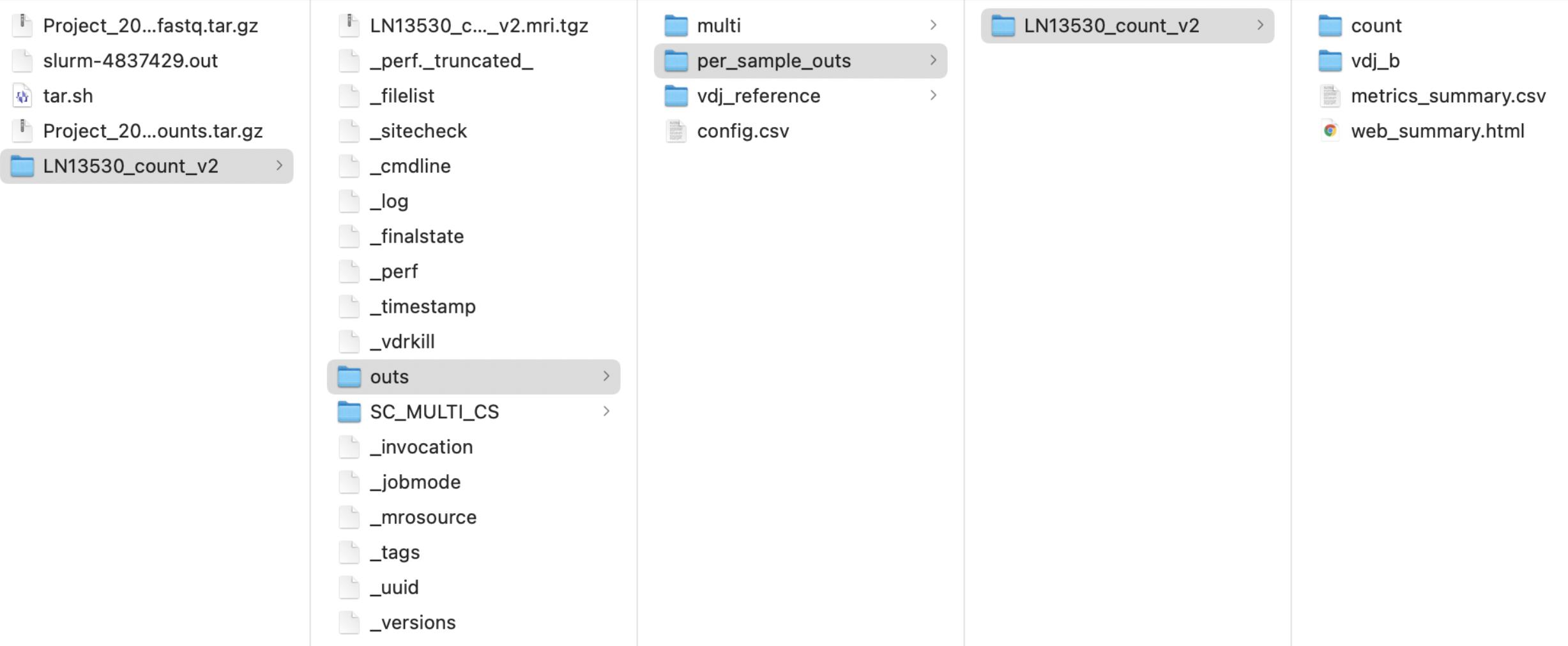
How to run cellranger count

```
path_to_cellranger/cellranger count --id=samplename(your choice) --transcriptome=path_to/refdata-gex-GRCh38-2020-A  
--fastqs=path_to_flowcell/out/outs/fastq_path --sample=sample_prefix
```

output



```
path_to_cellranger/cellranger-6.0.1/cellranger multi --id=samplename(your choice) --csv=multi-config.csv
```



Matrix output

With 3 files needed to completely describe each gene x cell matrix

- matrix.mtx.gz
- features.tsv.gz
- barcode.tsv.gz

Type	Description
Raw	gene-barcode matrices Contains every barcode from fixed list of known-good barcode sequences. This includes background and non-cellular barcodes.
Filtered	gene-barcode matrices Contains only detected cellular barcodes.

Web summary examples

LN13530_GEX

Summary

Analysis

11,597

Estimated Number of Cells

32,179

Mean Reads per Cell

1,665

Median Genes per Cell

Sequencing ?

Number of Reads 373,184,921

Number of Short Reads Skipped 0

Valid Barcodes 93.4%

Valid UMIs 99.8%

Sequencing Saturation 77.4%

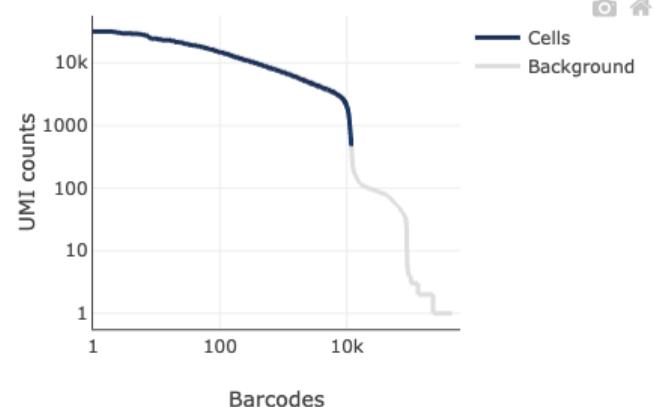
Q30 Bases in Barcode 95.5%

Q30 Bases in RNA Read 86.7%

Q30 Bases in UMI 94.7%

Cells ?

Barcode Rank Plot



Estimated Number of Cells 11,597

Fraction Reads in Cells 92.1%

Mean Reads per Cell 32,179

Median Genes per Cell 1,665

Total Genes Detected 20,470

Median UMI Counts per Cell 3,502

Mapping

Reads Mapped to Genome	91.6%
Reads Mapped Confidently to Genome	87.0%
Reads Mapped Confidently to Intergenic Regions	4.8%
Reads Mapped Confidently to Intronic Regions	10.1%
Reads Mapped Confidently to Exonic Regions	72.1%
Reads Mapped Confidently to Transcriptome	64.3%
Reads Mapped Antisense to Gene	4.5%

Sample

Sample ID	LN13530_GEX
Sample Description	
Chemistry	Single Cell 5' R2-only
Include introns	False
Reference Path	...ts/Roberta/refdata-gex-GRCh38-2020-A
Transcriptome	GRCh38-2020-A
Pipeline Version	cellranger-6.0.1

LN13530_GEX

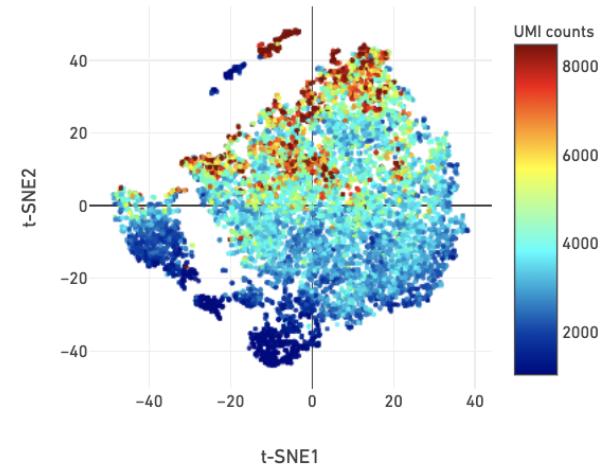
Summary

Analysis

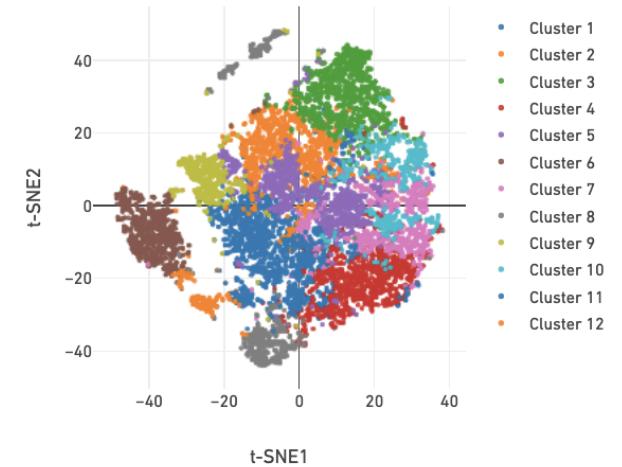
t-SNE Projection ?

Clustering Type: Graph-based ▼

t-SNE Projection of Cells Colored by UMI Counts



t-SNE Projection of Cells by Clustering



Top Features by Cluster (Log2 fold-change, p-value) ?

Feature		Cluster 1		Cluster 2		Cluster 3		Cluster 4		Cluster 5		Cluster 6		Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11	Cluster 12
ID	Name	L2FC	p-value	L2FC	p-value	L2FC	p-value												
ENSG00000111679	PTPN6	1.43	2e-4	0.16	1e+0	-0.93	9e-2	0.02	1e+0	-0.90	8e-1	0.15	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000158481	CD1C	1.19	7e-3	0.44	1e+0	-1.61	1e-4	-0.45	1e+0	-0.03	1e+0	-0.13	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000104903	LYL1	1.18	9e-3	0.20	1e+0	-0.54	7e-1	-0.25	1e+0	-0.05	1e+0	-0.14	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000216490	IFI30	1.16	1e-2	-0.00	1e+0	-0.44	9e-1	-0.79	1e+0	-0.47	1e+0	-0.12	1e+0	-0.11	1e+0	-0.11	1e+0	-0.11	1e+0
ENSG00000197043	ANXA6	1.12	2e-2	0.18	1e+0	-1.53	6e-4	0.12	1e+0	-0.10	1e+0	0.53	5e-1	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000142227	EMP3	0.96	1e-1	0.36	1e+0	-1.22	1e-2	0.38	1e+0	-0.33	1e+0	-0.19	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000137101	CD72	0.90	2e-1	0.25	1e+0	-0.71	4e-1	-0.50	1e+0	-0.11	1e+0	0.11	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000026297	RNASET2	0.87	2e-1	0.29	1e+0	-0.98	6e-2	0.38	1e+0	-0.19	1e+0	0.12	1e+0	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000162144	CYB561A3	0.73	6e-1	0.16	1e+0	-0.68	4e-1	-0.22	1e+0	-0.03	1e+0	0.36	8e-1	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0
ENSG00000173457	PPP1R14B	0.72	7e-1	0.04	1e+0	-0.18	1e+0	-0.12	1e+0	0.12	1e+0	-0.59	8e-1	-0.05	1e+0	-0.05	1e+0	-0.05	1e+0

Previous

Page 1 of 45

10 rows ▼

Next

Sample ⓘ

?

Library

?

Experimental
Design

?

Sample ID

Pipeline Version

LN13530_count_v2

cellranger-6.0.1

Gene Expression

VDJ-B ⓘ

Chemistry

Single Cell 5' R2-only

Include Introns

false

Reference Path

/exports/lgtc/projects/Roberta/refdata-gex-GRCh38-2020-A

Transcriptome

GRCh38-2020-A

Cells ⓘ

Cells	Median reads per cell	Median genes per cell	Total genes detected	Median UMI counts per cell
11,653	23,446	1,662	20,473	3,495

Mapping Metrics (Amongst Reads From Cells Assigned To Sample) ⓘ

Number of reads assigned to the sample	Mapped to genome	Confidently mapped to genome	Confidently mapped to transcriptome	Confidently mapped to intronic regions	Confidently mapped to exonic regions	Confidently mapped to intergenic regions	Confidently mapped antisense
316,261,767	92.26%	89.45%	68.39%	10.13%	75.09%	4.23%	3.17%

Understand the new web summary

Sample ⓘ



Library



Experimental



Design



Chemistry Single Cell 5' R2-only

Include Introns false

Reference Path /exports/lgtc/projects/Roberta/refdata-gex-GRCh38-2020-A

Transcriptome GRCh38-2020-A

Cell Statistics ⓘ

Physical library ID	Estimated number of cells	Mean reads per cell
GEX_1	11,653	32,025

Sequencing Metrics ⓘ

Fastq ID	Number of reads	Number of short reads skipped	Q30 barcodes	Q30 UMI	Q30 RNA read
LN13530_GEX	373,184,921	0	95.5%	94.7%	86.7%

Mapping Metrics (Amongst All Reads in Library) ⓘ

Physical library ID	Number of reads in the library	Mapped to genome	Confidently mapped to genome	Confidently mapped to transcriptome	Confidently mapped to intronic regions	Confidently mapped to exonic regions	Confidently mapped to intergenic regions	Confidently mapped antisense
GEX_1	373,184,921	91.61%	87.01%	64.28%	10.07%	72.11%	4.83%	4.47%

Metrics Per Physical Library ⓘ

Physical library ID	Number of reads	Valid barcodes	Valid UMIs	Sequencing saturation	Fraction reads in cells	Mean reads per cell
GEX_1	373,184,921	93.39%	99.78%	77.40%	92.32%	32,025

✉ Understand the new web summary

Metric	Description
Estimated Number of Cells	The number of barcodes associated with cell-containing partitions
Mean Reads per Cell	The total number of sequenced reads divided by the estimated number of cells.
Median Genes per Cell	The median number of genes detected (with nonzero UMI counts) across all cell-associated barcodes.
Number of Reads	Total number of sequenced reads.
Valid Barcodes	Fraction of reads with cell-barcodes that match the whitelist.
Reads Mapped to Genome	Fraction of reads that mapped to the genome.
Reads Mapped Confidently to Genome	Reads Mapped Confidently to Genome.
Reads Mapped Confidently to Transcriptome	Fraction of reads that mapped to a unique gene in the transcriptome with a high mapping quality score
Reads Mapped Confidently to Exonic Regions	Fraction of reads that mapped to the exonic regions of the genome with a high mapping quality score
Reads Mapped Confidently to Intronic Regions	Fraction of reads that mapped to the intronic regions of the genome with a high mapping quality score
Reads Mapped Confidently to Intergenic Regions	Fraction of reads that mapped to the intergenic regions of the genome with a high mapping quality score
Reads Mapped Antisense to Gene	Fraction of reads confidently mapped to the transcriptome, but on the opposite strand of their annotated gene.
Sequencing Saturation	The fraction of reads originating from an already-observed UMI. This is a function of library complexity and sequencing depth.
Q30 Bases in Barcode	Fraction of bases with Q-score at least 30 in the cell barcode sequences.
Q30 Bases in RNA Read	Fraction of bases with Q-score at least 30 in the RNA read sequences.
Q30 Bases in Sample Index	Fraction of bases with Q-score at least 30 in the sample index sequences.
Q30 Bases in UMI	Fraction of bases with Q-score at least 30 in the UMI sequences.
Fraction Reads in Cells	The fraction of cell-barcoded, confidently mapped reads with cell-associated barcodes.
Total Genes Detected	The number of genes with at least one UMI count in any cell.
Median UMI Counts per Cell	The median number of total UMI counts across all cell-associated barcodes.

Antibody Sequencing

Version

Number of Reads

Total number of Antibody library reads.

Valid Barcodes

Fraction of Antibody library reads with a barcode found in or corrected to one that is found in the whitelist.

Valid UMIs

Fraction of Antibody library reads with valid UMIs.

Sequencing Saturation

The fraction of Antibody library reads originating from an already-observed UMI. This is a function of library complexity and sequencing depth. More specifically, this is the fraction of confidently mapped, valid cell-barcode, valid UMI reads that had a non-unique (cell-barcode, UMI, CRISPR feature barcode).

Q30 Bases in Barcode

Fraction of Antibody library cell barcode bases with Q-score ≥ 30 , excluding very low quality/no-call ($Q \leq 2$) bases from the denominator.

Q30 Bases in Antibody Read

Fraction of Antibody library read bases with Q-score ≥ 30 , excluding very low quality/no-call ($Q \leq 2$) bases from the denominator. This is Read 2 for the Single Cell 3' v3 and Single Cell 5' chemistries.

Q30 Bases in UMI

Fraction of Antibody library UMI bases with Q-score ≥ 30 , excluding very low quality/no-call ($Q \leq 2$) bases from the denominator.

Antibody Application

Fraction Antibody Reads

Fraction of Antibody library reads that contain a recognized antibody barcode.

Fraction Antibody Reads Usable

Fraction of Antibody library reads that contain a recognized antibody barcode, a valid UMI, and a cell-associated barcode.

Antibody Reads Usable per Cell

Number of Antibody library reads usable divided by the number of cell-associated barcodes.

Fraction Reads in Barcodes with High UMI Counts

Fraction of Antibody library reads that was lost after removing barcodes with unusually high UMI counts (possibly aggregates).

Fraction Unrecognized Antibody

Among all Antibody library reads, the fraction with an unrecognizable antibody barcode.

Antibody Reads in Cells

Among Antibody library reads with a recognized antibody barcode, a valid UMI, and a valid barcode, the fraction associated with cell-containing partitions.

Median UMIs per Cell (summed over all recognized antibody barcodes)

Median UMIs per Cell (summed over all recognized antibody barcodes).

Sample ID
202102750_count

Pipeline Version
cellranger-6.0.1

Sample



Gene Expression

Antibody

Library



Chemistry Single Cell 5' R2-only

Experimental Design



Include Introns false

Reference Path /exports/lgtc/projects/Roberta/refdata-gex-mm10-2020-A/

Transcriptome mm10-2020-A

Cells

Cells	Median reads per cell	Median genes per cell	Total genes detected	Median UMI counts per cell
8,325	21,768	1,318	18,368	3,221

Mapping Metrics (Amongst Reads From Cells Assigned To Sample)

Number of reads assigned to the sample	Mapped to genome	Confidently mapped to genome	Confidently mapped to transcriptome	Confidently mapped to intronic regions	Confidently mapped to exonic regions	Confidently mapped to intergenic regions	Confidently mapped antisense
242,992,392	91.97%	89.35%	72.01%	6.82%	77.32%	5.21%	2.63%

t-SNE Projection

Clustering Type: Graph-based

Sample



Library

Experimental
Design

Cell Statistics

Physical library ID	Estimated number of cells	Mean reads per cell
GEX_1	8,325	35,694

Sequencing Metrics

Fastq ID	Number of reads	Number of short reads skipped	Q30 barcodes	Q30 UMI	Q30 RNA read
202102750_GEX	297,150,101	0	95.7%	95.1%	86.1%

Mapping Metrics (Amongst All Reads in Library)

Physical library ID	Number of reads in the library	Mapped to genome	Confidently mapped to genome	Confidently mapped to transcriptome	Confidently mapped to intronic regions	Confidently mapped to exonic regions	Confidently mapped to intergenic regions	Confidently mapped antisense
GEX_1	297,150,101	89.60%	86.62%	64.63%	7.64%	71.84%	7.13%	4.66%

Metrics Per Physical Library

Physical library ID	Number of reads	Valid barcodes	Valid UMIs	Sequencing saturation	Fraction reads in cells	Mean reads per cell
GEX_1	297,150,101	90.63%	99.77%	78.84%	94.39%	35,694

Understand the new web summary

Metric	Exon-only	Intron-mode
Estimated Number of Cells	11,996	11,984
Mean Reads per Cell	41,379	41,421
Fraction Reads in Cells	96.0%	96.4%
Reads Mapped Confidently to Transcriptome	52.7%	77.7%
Median Genes per Cell	2,049	3,268 (+59%)
Median UMI Counts per Cell	6,896	10,186 (+48%)
Total Genes Detected	25,589	30,351 (+19%)

Cell Ranger Cell Statistic Definitions

Estimated Number of Cells	The number of barcodes identified by the cell-calling algorithm as containing a cell.
Mean Reads per Cell	The total number of sequenced read pairs divided by the number of cell-associated barcodes.
Fraction Reads in Cells	The fraction of valid-barcode, valid-UMI, confidently-mapped-to-transcriptome reads with cell-associated barcodes.
Reads Mapped Confidently to Transcriptome	Fraction of reads that mapped to a unique gene in the transcriptome. The read must be consistent with annotated splice junctions. These reads are considered for UMI counting.
Median Genes per Cell	Median number of read pairs sequenced from the cells assigned to this sample (at least one UMI count detected).
Median UMI Counts per Cell	Median number of UMIs obtained from the cells assigned to this sample.
Total Genes Detected	The number of genes with at least one UMI count in any cell.

Sample



Chemistry Single Cell 5' R2-only

Library



Include Introns false

Experimental



Reference Path /exports/lgtc/projects/Roberta/refdata-gex-mm10-2020-A/

Design



Transcriptome mm10-2020-A

Cell Statistics

Physical library ID	Estimated number of cells	Mean reads per cell
ABC_1	8,325	5,094

Sequencing Metrics

Fastq ID	Number of reads	Number of short reads skipped	Q30 barcodes	Q30 UMI	Q30 RNA read
202102750_HTO	42,409,538	0	95.9%	94.9%	89.2%

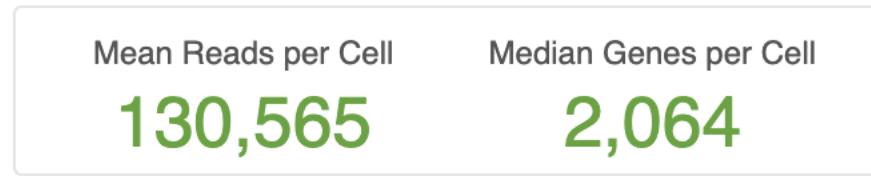
Metrics Per Physical Library

Physical library ID	Number of reads	Valid barcodes	Valid UMIs	Fraction reads in cells	Mean reads per cell	Fraction antibody reads	Fraction antibody reads usable	Fraction unrecognized antibody	Fraction antibody reads in aggregate barcodes
ABC_1	42,409,538	97.86%	100.00%	51.53%	5,094	96.68%	48.79%	3.32%	20.70%

Understand the new web summary

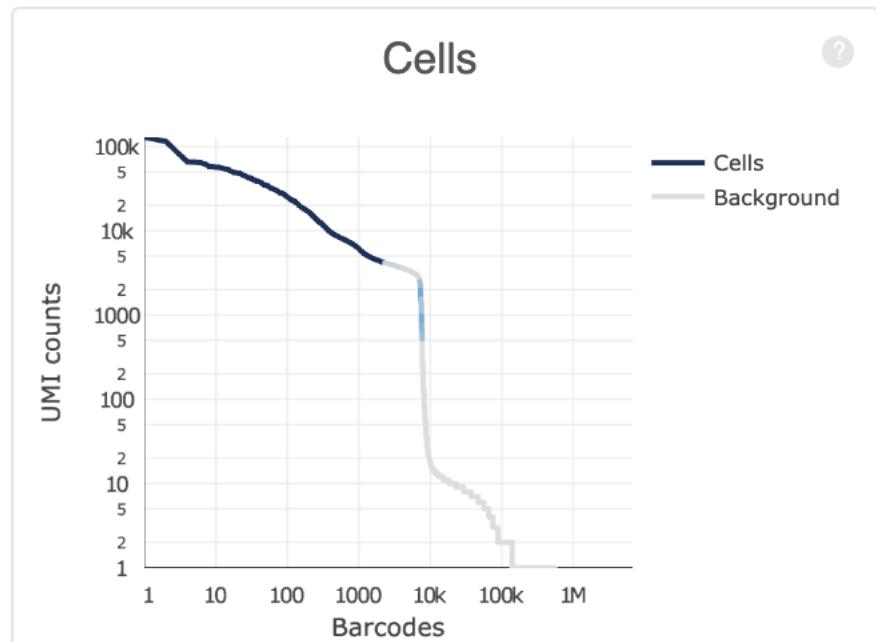
The analysis detected some issues. [Details »](#)

Alert	Value	Detail
⚠ Low Fraction Reads in Cells	53.4%	Ideal > 70%. Application performance may be affected. Many of the reads were not assigned to cell-associated barcodes. This could be caused by high levels of ambient RNA or by a significant population of cells with a low RNA content, which the algorithm did not call as cells. The latter case can be addressed by inspecting the data to determine the appropriate cell count and using --force-cells.



Sequencing

Number of Reads	333,334,060
Valid Barcodes	97.5%
Sequencing Saturation	74.3%
Q30 Bases in Barcode	96.1%
Q30 Bases in RNA Read	90.8%
Q30 Bases in Sample Index	94.8%
Q30 Bases in UMI	93.3%



Estimated Number of Cells **2,553**

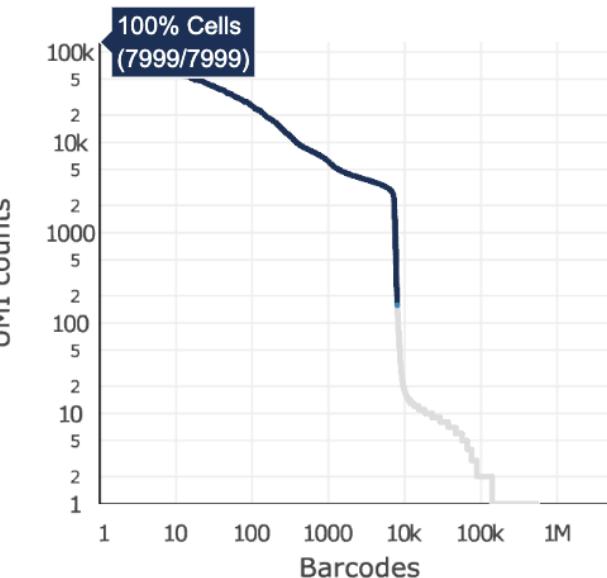
Fraction Reads in Cells **53.4%**

Mean Reads per Cell	130,565
Median Genes per Cell	2,064
Total Genes Detected	18,966
Median UMI Counts per Cell	5,148

Estimated Number of Cells

8,000

Cells



Mean Reads per Cell

41,666

Median Genes per Cell

1,586

Sequencing

Number of Reads

333,334,060

Valid Barcodes

97.5%

Sequencing Saturation

74.3%

Q30 Bases in Barcode

96.1%

Q30 Bases in RNA Read

90.8%

Q30 Bases in Sample Index

94.8%

Q30 Bases in UMI

93.3%

Mapping

Reads Mapped to Genome

94.9%

Reads Mapped Confidently to Genome

93.0%

Reads Mapped Confidently to Intergenic Regions

5.0%

Reads Mapped Confidently to Intronic Regions

38.4%

Reads Mapped Confidently to Exonic Regions

49.6%

Reads Mapped Confidently to Transcriptome

45.7%

Reads Mapped Antisense to Gene

1.4%

Sample

Name

_GEX_count_8k

Description

GRCh38

Transcriptome

Single Cell 3' v3

Chemistry

3.0.0

Cell Ranger Version

Forcing the number of cells

Conclusions

Always perform QC of your libraries!

Be aware of library specifics → critical mindset!