



SMRT<sup>®</sup> Link  
Kinnex<sup>™</sup> single-cell  
troubleshooting  
guide (v13.1  
and later)



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## Introduction

This document describes the metrics generated by the **Read Segmentation** and **Single-Cell Iso-Seq**® workflow in SMRT Link v13.1 and later. The document also describes possible issues that can occur when using the **Kinnex single-cell RNA** kit for both supported and unsupported use cases.

**Kit compatibility:** The workflows described in this document are for single-cell Iso-Seq data generated using Kinnex single-cell RNA kit. However, it can also be used to analyze data generated using the MAS-Seq for 10x Single Cell 3' kit (no longer available for purchase). There are multiple single-cell platforms that are compatible with the Kinnex kits, however, the Single-Cell Iso-Seq workflow is designed to work with 10x single-cell cDNA (Next GEM Universal and GEM-X Universal). To learn more about the general Kinnex technology, visit <https://pacb.com/kinnex>.

- Example data sets are available [here](#).
- Additional command-line information, example commands, and suggestions for tertiary analyses are described [here](#).

## SMRT Link read segmentation

The SMRT Link Read Segmentation workflow can be invoked either as a standalone Data Utility workflow, or in combination with single-cell Iso-Seq as an Analysis workflow. For Kinnex single-cell users using the **Kinnex single-cell RNA** kit, **Read Segmentation and Single-Cell Iso-Seq** is the recommended workflow.

Read segmentation deconcatenates HiFi reads into segmented reads (S-reads) based on segmentation adapters, using the command-line `skera` tool. (See [here](#) for details.)

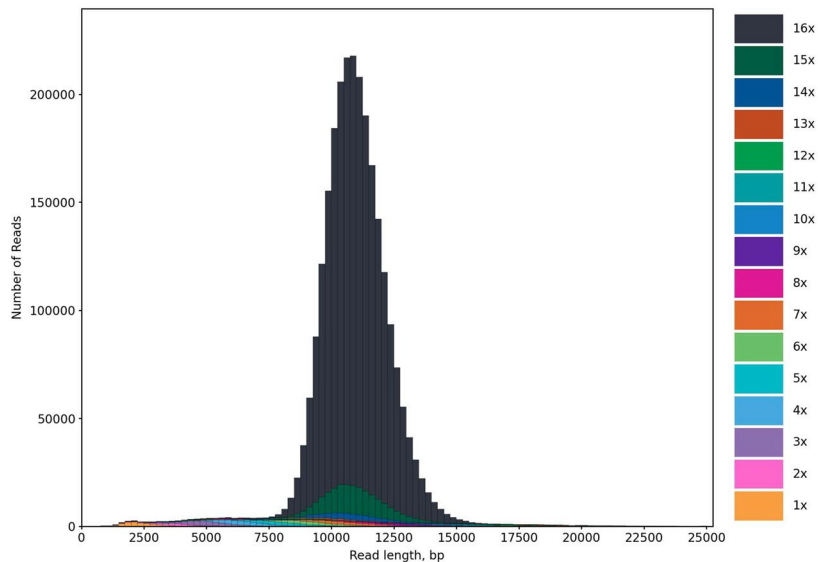
The Kinnex single-cell RNA kit enriches for full (16-fold) arrays, while most single-cell cDNA libraries are 600-1000 bp. Therefore, the percentage of full array and concatenation factors should have typical values as shown below.

Metric	Explanation	Typical value
Reads	Number of HiFi reads	Depends on sequencing yield
S-reads	Number of segmented reads	Depends on HiFi read yield and concatenation success
Mean Length of S-reads	Mean read length of S-reads	600-800bp for most single-cell cDNA
Percent of Reads with Full Arrays	Percent of HiFi reads with full Kinnex arrays	85-90%
Mean Array Size	Concatenation factor	~15.xx

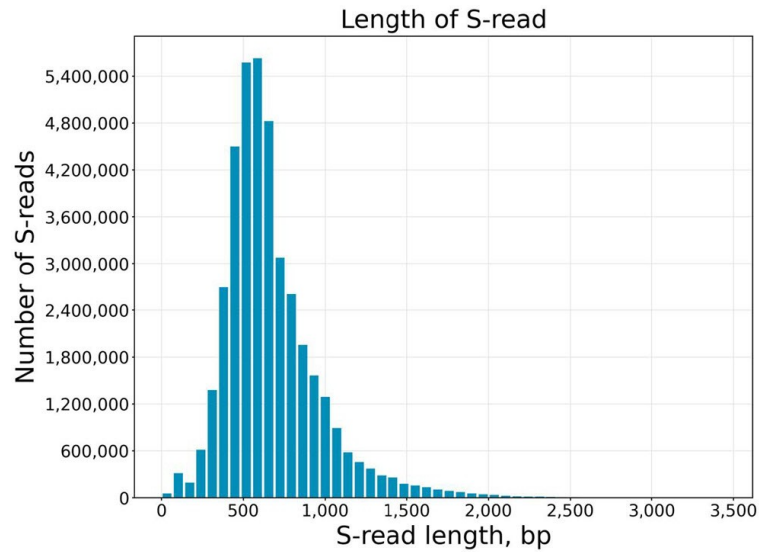
**Read Segmentation**

Value	Analysis Metric	
2,622,891	Reads	← Input # HiFi reads. Depends on loading (P1), Pol RL, HiFi conversion rate
40,131,832	Segmented reads (S-reads)	
672	Mean length of S-reads	← Depends on input 10x cDNA size, but generally 600-800 bp
86.32 %	Percent of reads with full arrays	← Regardless of input cDNA size and input reads, should be at least ~8x%
15.30	Mean array size (concatenation factor)	← Regardless of input cDNA size and input reads, should always be ~15.xx

A clean peak between 10,000 – 14,000 bp indicates good Kinnex array formation and successful enrichment of full arrays:



S-read read length should largely reflect the original single-cell cDNA library size:



## SMRT Link Single-Cell Iso-Seq workflow: Read statistics

cDNA primers and polyA tails are removed from S-reads, then UMI/BC are extracted and reads are deduplicated. This is performed using the command `isoseq3 tag/refine/correct/groupdedup`. (See [here](#) for the high-level workflow.)

Metric	Explanation	Typical value
Reads	Number of S-reads	Depends on sequencing yield
Read Type	CCS or SEGMENT	CCS or SEGMENT
Reads with 5' and 3' Primers with Extracted UMIs and Barcodes	Full-Length (FL) tagged reads	>95% of reads should be FL tagged
Non-Concatemer Reads with 5' and 3' Primers and PolyA Tail	Full-Length Non-Concatemer (FLNC) tagged reads	>90% of reads should be FLNC tagged
FLNC Reads with Valid Barcodes	FLNC reads matching a barcode white list	>90% of reads should match barcodes in the white list
FLNC Reads with Valid Barcodes, Corrected	FLNC reads matching the barcode white list after correction	>90% of reads should match barcodes in the whitelist after correction
Reads After Barcode Correction and UMI Deduplication	Deduplicated reads	Deduplicated read yield depends on the 10x library complexity and PCR duplication rate

Value	Analysis Metric	
40,131,832	Reads	← Input # of S-reads, from Read Segmentation
SEGMENT	Read Type	
39,557,330	Reads with 5' and 3' Primers with extracted UMIs and Barcodes	← Most S-reads should have the expected cDNA primers
37,693,809	Non-Concatamer Reads with 5' and 3' Primers and Poly-A Tail (FLNC reads)	← Most FL reads should have polyA tails and are not concatemers
36,526,033	FLNC Reads with Valid Barcodes	← Most FLNC reads should have valid barcodes
37,634,585	FLNC Reads with Valid Barcodes, corrected	
23,883,685	Reads after Barcode Correction and UMI Deduplication	← The # of deduplicated reads depends on library complexity. The fewer the deduplicated reads, the more PCR duplicates there are

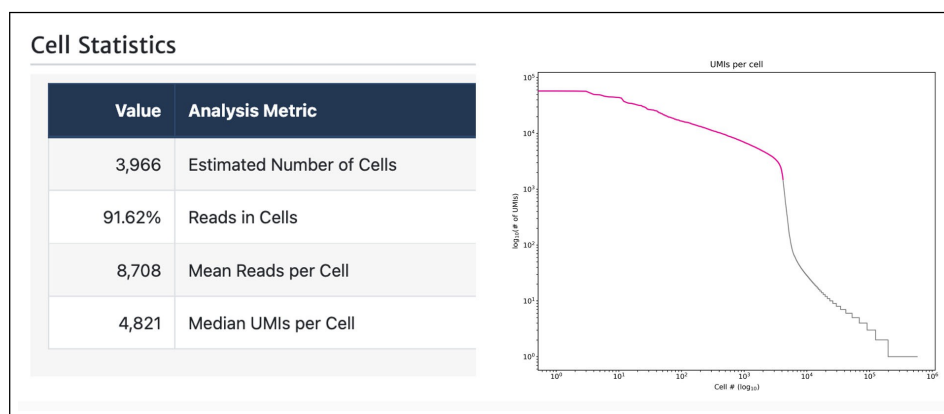
## SMRT Link Single-Cell Iso-Seq workflow: Cell statistics

The number of estimated cells ("real cells") varies by experiment. The estimation is performed using the `isoseq3 bcstats` command. (See [here](#) for information.)

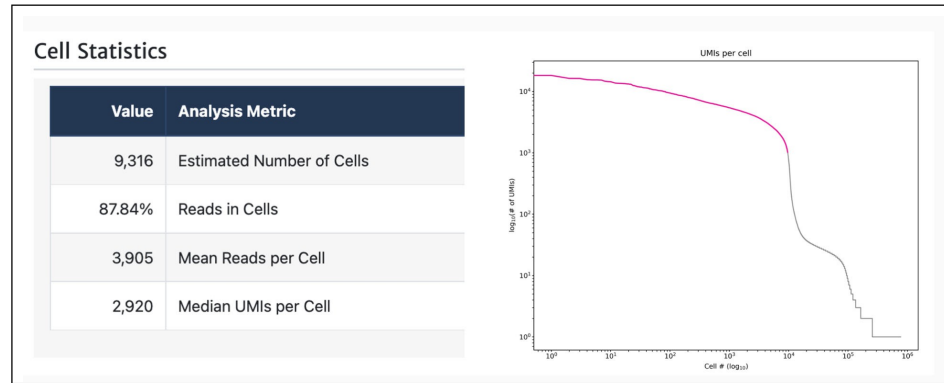
Metric	Explanation	Typical value
Estimated Number of Cells	The number of real cells	Depends on the 10x library
Reads in Cells	The percent of reads in real cells	>85%
Mean Reads per Cell	The mean reads per real cell	Depends on the 10x library and read yield
Median UMIs per Cell	The median UMI per real cell	Depends on the 10x library, read yield, and PCR duplication rate

The estimated number of cells, mean reads per cell and median UMIs per cell are highly dependent on the single-cell library and sample complexity. If you suspect that the cell estimation is incorrect using the default `knee` method for `isoseq3 correct`, the cells can be re-estimated using the alternative `percentile` method. (See [here](#) for details.)

### Example 1: PBMC 5k cells - Cell statistics



## Example 2: PBMC 10k cells - Cell statistics



## SMRT Link Single-Cell Iso-Seq workflow: Transcript statistics

Deduplicated reads are mapped to a genome, classified and filtered using `pigeon` software (SQANTI3). This is performed using the command `pbmm2/iseq3 collapse/pigeon`. (See [here](#) for information.)

Metric	Explanation	Typical value
FLNC Reads Mapped Confidently to Genome	FLNC reads (before deduplication) mapped to the genome. <sup>a</sup>	~80%
FLNC Reads Mapped Confidently to Transcriptome	FLNC reads (before deduplication) mapped to transcriptome <sup>b</sup>	30-50%
Total Unique Genes	Total unique genes <b>before</b> <code>pigeon</code> filtering <sup>c</sup>	Sample-dependent
Total Unique Genes, filtered	Total unique genes <b>after</b> <code>pigeon</code> filtering <sup>c</sup>	Sample-dependent
Total Unique Genes, known genes only	Total unique known genes <b>before</b> <code>pigeon</code> filtering <sup>c</sup>	Sample-dependent
Total Unique Genes, filtered, known genes only	Total unique known genes <b>after</b> <code>pigeon</code> filtering <sup>c</sup>	Sample-dependent
Total Unique Transcripts	Total unique transcripts <b>before</b> <code>pigeon</code> filtering	Sample-dependent
Total Unique Transcripts, filtered	Total unique transcripts <b>after</b> <code>pigeon</code> filtering	Sample-dependent
Total Unique Transcripts, known transcripts only	Total unique known transcripts <b>before</b> <code>pigeon</code> filtering	Sample-dependent
Total Unique Transcripts, filtered, known transcripts only	Total unique known transcripts <b>after</b> <code>pigeon</code> filtering	Sample-dependent

- a. FLNC reads mapped to the genome after running `iseq3 collapse`. Though actual mapping is done with deduplicated reads, UMI count is summarized post-mapping to reflect the pre-deduplicated FLNC count. Note that `iseq3 collapse` filters for reads that map chimerically or map with low identity, so if there are cancer fusion genes or genes not well represented in the genome, they would be **excluded** at this step. In general, one should expect most (~80%) FLNC reads to map to the genome, even if they end up mapping to, say, intergenic regions.

- b. FLNC reads mapped to known genes (known or novel isoforms) after `pigeon classify` and `pigeon filter`. This number more likely represents the “number of usable reads” that actually go into a standard single-cell analysis. This number includes ribosomal/mitochondrial genes. It is typical to see 30-50% FLNC reads map to the transcriptome, which is consistent with equivalent 10x short read sequencing data. Most of the non-transcriptomic but genomically-mapped reads are attributed to intergenic regions and are filtered out by `pigeon filter`.
- c. It is typical to see a very high number of “total number of genes/transcripts” before `pigeon filter`. This is due to the high number of loci that are intergenic and still being assigned a “novel gene” status before `pigeon filter`.

Transcript Statistics

Value	Analysis Metric
30,434,177	FLNC Reads Mapped Confidently to Genome
15,231,566	FLNC Reads Mapped Confidently to Transcriptome
1,517,432	Total Unique Genes
31,913	Total Unique Genes, filtered
29,849	Total Unique Genes, known genes only
21,596	Total Unique Genes, filtered, known genes only
2,487,669	Total Unique Transcripts
287,853	Total Unique Transcripts, filtered
835,769	Total Unique Transcripts, known transcripts only
276,025	Total Unique Transcripts, filtered, known transcripts only

FLNC reads mapped to the genome after running `isoseq3 collapse` (dedup reads were mapped but expand it back to reflect the pre-deduplicated FLNC count)

Note: `isoseq3 collapse` filters for reads that map chimerically or map with low identity, so if there are cancer fusion genes or genes not well represented in the genome, they'd be excluded at this step

FLNC reads mapped to known genes (known or novel isoforms) after `pigeon classify` and `pigeon filter`. Think of this as the “number of usable reads” that actually go into a standard single-cell analyses.

Note: this number includes ribosomal/mitochondrial genes

After `pigeon` filtering, the number of genes/isoforms per cell:

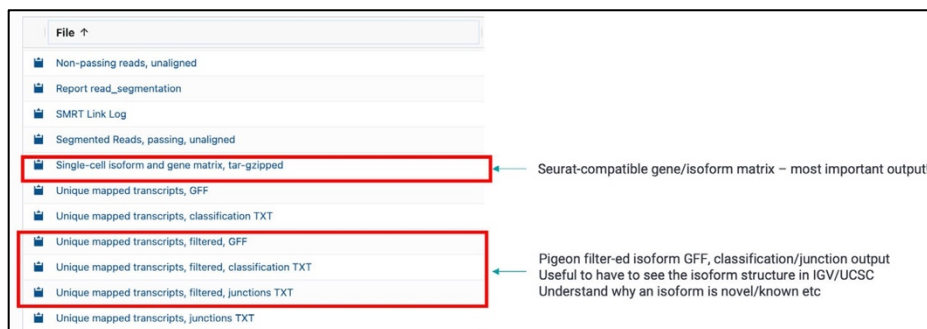
Transcript Summary, filtered

Value	Analysis Metric
705	Median Genes per Cell
700	Median Genes per Cell, known genes only
821	Median Transcripts per Cell
816	Median Transcripts per Cell, known transcripts only
31,913	Total Unique Genes
21,596	Total Unique Genes, known genes only
287,853	Total Unique Transcripts
276,025	Total Unique Transcripts, known transcripts only

Probably the most important stats for users – this is essentially the “sequencing depth” per cell. How much one can get per cell will depend on:

- S-read yield
- Number of cells
- Sample type
- Library complexity (PCR duplicate rate)

## SMRT Link Read Segmentation and Single-Cell Iso-Seq workflow: File downloads



## Possible issues when using the Kinnex single-cell RNA kit for supported use cases

The currently-supported use case for the Kinnex single-cell kit is a single-cell library produced using the 10x Next GEM or GEM-X kits (5' or 3'), with a typical use case of up to 20,000 target cell recovery.

Observed issue	Likely cause	Solution
<ul style="list-style-type: none"> <li>Good concatenation factor</li> <li>Low S-read yield</li> </ul>	Low P1 loading or HiFi conversion	Perform additional sequencing
<ul style="list-style-type: none"> <li>Good S-read yield</li> <li>Poor FLNC yield and beyond</li> </ul>	Using a different version of the 10x 3' or 5' kit (SL supports barcode whitelist for 3' v3.1 and 5' v2)	Reanalyze with proper cDNA primer, UMI/BC design and barcode white list. Additional 10x cDNA primers and barcode white list can be found <a href="#">here</a> .
<ul style="list-style-type: none"> <li>Good S-read yield</li> <li>Good cell statistics</li> <li>Poor read mapping and low gene counts</li> </ul>	The wrong reference was selected.	Choose correct reference genome and annotation. SMRT Link supports <b>only</b> human and mouse reference genome + Gencode annotation (available <a href="#">here</a> ). If using different genomes or annotations, refer to the <a href="#">pigeon</a> documentation for command line analysis. (See <a href="#">here</a> for details.)
<ul style="list-style-type: none"> <li>Good S-read yield</li> <li>Poor cell recovery</li> </ul>	The algorithm underestimated the number of cells.	Reanalyze using the <a href="#">percentile</a> method in SMRT Link or using the command line. (See <a href="#">here</a> for details.)
<ul style="list-style-type: none"> <li>Analysis experienced an error, but was able to recover and complete successfully; High Barcode Errors</li> </ul>	Incorrect barcode white list	Reanalyze using the correct barcode white list. The error message <b>Analysis experienced an error, but was able to recover and complete successfully; High Barcode Errors</b> indicates that the barcode white list provided is <b>incorrect</b> . Note that SMRT Link expects a barcode white list that is reverse-complemented, which is not how the 10x white list is typically provided. A list of common barcode white list in reverse-complement can be found <a href="#">here</a> .

## Troubleshooting Example 1: Wrong reference selected, poor gene/transcript recovery

Inputs	Data Type	Name	Import Complete
	BarcodeSet	Barcode Sets: 10x Chromium single cell 3' cDNA primers	Yes
	BarcodeSet	Barcode Sets: MAS-Seq Adapter v1 (MAS16)	Yes
	ConsensusReadSet	HiFi Reads: JacksonLab_1-Cell4 (CCS)	Yes
	ReferenceSet	References: Human Genome hg38, with Gencode v39 annotations	Yes

Transcript Summary, filtered

Value	Analysis Metric
32	Median Genes per Cell
32	Median Genes per Cell, known genes only
34	Median Transcripts per Cell
34	Median Transcripts per Cell, known transcripts only
2,394	Total Unique Genes
2,359	Total Unique Genes, known genes only
10,781	Total Unique Transcripts
10,731	Total Unique Transcripts, known transcripts only

## Correct reference selected, good gene/transcript recovery

Inputs	Data Type	Name	Import Complete
	BarcodeSet	Barcode Sets: 10x Chromium single cell 3' cDNA primers	Yes
	ConsensusReadSet	HiFi Reads: JacksonLab_1-Cell4 (CCS) Segmented Reads	Yes
	ReferenceSet	References: Mouse Genome mm39, with Gencode vM28 annotati...	Yes

Transcript Summary, filtered

Value	Analysis Metric
757	Median Genes per Cell
751	Median Genes per Cell, known genes only
842	Median Transcripts per Cell
837	Median Transcripts per Cell, known transcripts only
28,754	Total Unique Genes
20,446	Total Unique Genes, known genes only
340,640	Total Unique Transcripts
330,490	Total Unique Transcripts, known transcripts only

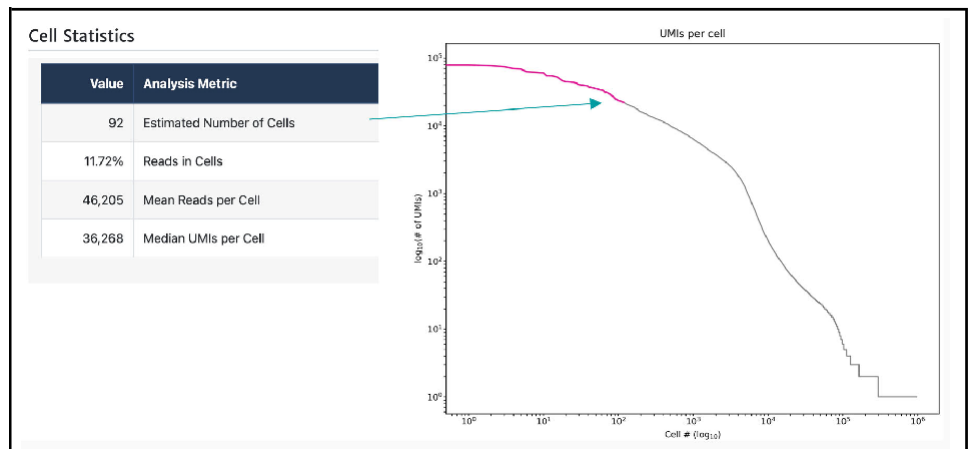
## Troubleshooting Example 2: Underestimating the number of cells

If you generated matching short read data or have an expected target cell recovery, you might identify cases in which the cell barcode calling algorithm **underestimated** the number of cells. This affects:

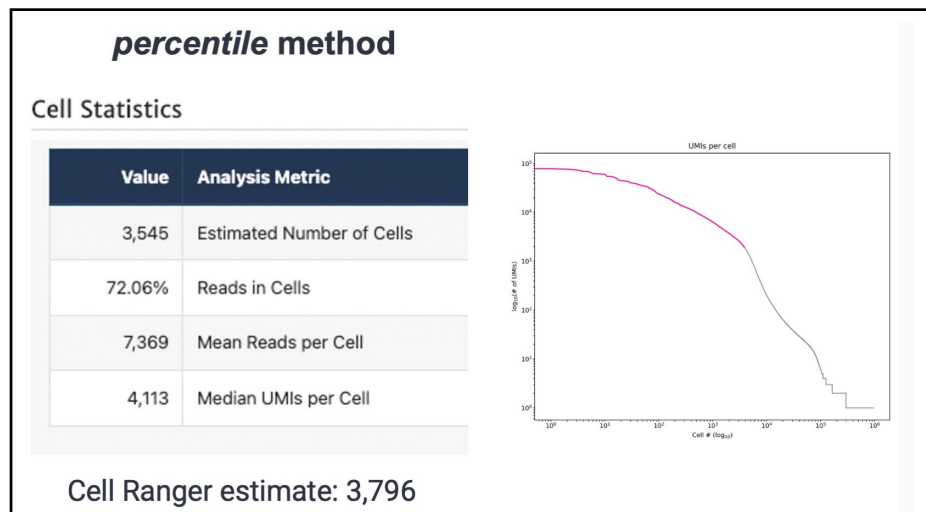
- Cell statistics
- Transcript statistics
- Output count matrix

It does **not** affect:

- Segmentation statistics
- Read statistics



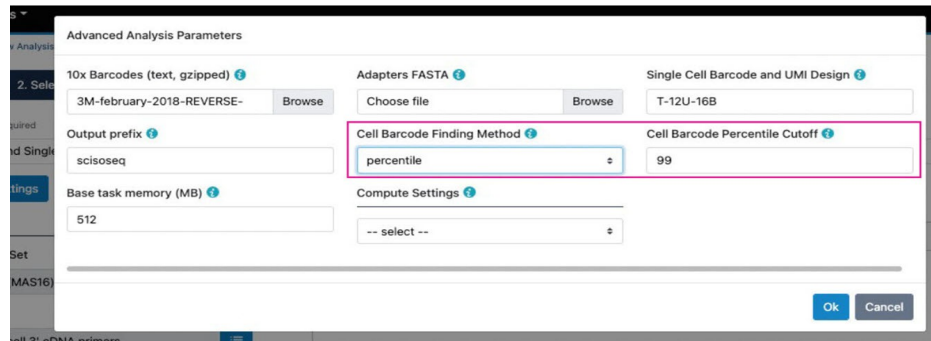
In most cases, the `knee` method is successful in estimating the number of real cells. Following are examples where the `knee` method was **not** successful, and the `percentile` method (with 97% or 99% cutoff) was used to achieve cell recovery.



Correct estimation of cells increases the number of usable FLNC.

Metric	Percentile	Knee
FLNC Reads Mapped Confidently to Genome	26,220,947	4,005,710
FLNC Reads Mapped Confidently to Transcriptome	9,105,973	2,089,836
Median Genes per Cell	239	3,888
Median Genes per Cell, known genes only	235	3,872
Median Transcripts per Cell	271	5,750
Median Transcripts per Cell, known transcripts only	269	5,726
Total Unique Genes	33,038	19,152
Total Unique Genes, known genes only	24,087	17,265
Total Unique Transcripts	336,099	118,066
Total Unique Transcripts, known transcripts only	326,154	116,022

SMRT Link supports the optional **percentile** method:



## Possible issues when using the Kinnex single-cell RNA kit for unsupported use cases

The following are **unsupported use cases** for the Kinnex single-cell RNA kit that are commonly observed. Note that PacBio **cannot** offer official support for library preparations, sequencing, or analyses for use of the kit in unsupported scenarios including those described below. The unsupported

use cases described herein have not been validated by PacBio® and are provided as-is and without any warranty. Use of these unsupported use cases is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential for use of their samples for analysis using the PacBio system. If any of part of these unsupported use cases is to be used in a production environment, it is the responsibility of the end user to perform the required validation.

Observed issue	Likely cause	Solution
<ul style="list-style-type: none"> <li>• Good S-read and FLNC yield</li> <li>• Poor FLNC with barcodes and beyond.</li> </ul>	Using the Kinnex single-cell kit with a Visium (spatial) library	Rerun the analysis using (1) cell barcode list; (2) barcode and UMI design.

## Using SMRT Link v13.1 and later with a Visium sample

NOTE: SMRT Link only supports 10x Visium but not Visium HD analysis. For Visium HD analysis, refer to 10x knowledge base and [isoseq.how](https://www.illumina.com/knowledgebase/articles/10x-iso-seq-how-to-use-visium-hd-analysis.html).

Visium samples have the exact same molecular structure as standard 10x 3' kit; the main inputs are identical to 3' analysis.

In the Read Segmentation and Single-Cell Iso-Seq's **Advanced Parameters** dialog, change **10x barcodes** to **Visium** barcodes (~5000 spots). Note that "cells" are basically spots if using SMRT Link to analyze Visium data.

The screenshot shows the 'Advanced Analysis Parameters' dialog box. The '10x Barcodes (text, gzipped)' field is highlighted with a pink box and contains the text 'visium-v1.RC.txt.gz'. Other fields include 'Adapters FASTA' (Choose file), 'Single Cell Barcode and UMI Design' (T-12U-16B), 'Output prefix' (scisoseq), 'Base task memory (MB)' (512), and 'Compute Settings' (C4\_P50). There are 'Ok' and 'Cancel' buttons at the bottom right.

### Example unsupported use case: Kinnex single-cell RNA kit with 10x Visium (spatial) library

The Kinnex single-cell RNA kit can work directly with Visium libraries **without** modification. Only the SMRT Link parameters require changing

## Incorrect parameters for Kinnex Visium unsupported use case

**Warning** Analysis experienced an error, but was able to recover and complete successfully.  
High Barcode errors: [isoseq] barcode correction ALARM: Missing fraction %99 > threshold 25% (task: pb\_sc\_isoseq.isoseq\_correct-0-a1)

▼ Analysis Parameters

**Adapters FASTA**

**Base task memory (MB)** 512

**Single Cell Barcode and UMI Design** T-12U-16B

**Output prefix** scisoseq

**10x Barcodes (text, gzipped)** /pbi/smrlink/smrlink-alpha/smrlink/current/bundles/smrlinkub/current/private/pacbio/barcodes/10X\_Barcodes/3M-february-2018-REVERSE-COMPLEMENTED.txt.gz

When the barcode white list is incorrect, SMRT Link displays a warning in the barcode correction step.

## Using SMRT Link v13.1 and later with GEM-X libraries

The 10x GEM-X libraries have the same adapter sequences as the prior versions. However, because the UMI and barcodes have changed, minor changes are required in running SMRT Link.

Download the 10x GEM-X barcodes from [here](#). In SMRT Link, change the parameters to upload the appropriate barcode whitelist and change the UMI/BC design to the following:

Advanced Analysis Parameters

10x Barcodes for 3' (text, gzipped) ⓘ

3M-3pgex-may-2023.REVCOMP.txt Browse

Upload GEM-X whitelist for 3'

10x Barcodes for 5' (text, gzipped) ⓘ

3M-5pgex-jan-2023.txt.gz Browse

Upload GEM-X whitelist for 5'

Single Cell Barcode and UMI Design for 3' ⓘ

T-12U-16B

No change for 3' in GEM-X

Single Cell Barcode and UMI Design for 5' ⓘ

16B-12U-13X-T Change UMI to 12bp  
Use 13X to remove TSO + (rG)s

output\_prefix ⓘ

scisoseq

Cell Barcode Finding Method ⓘ

knee ⇅

Cell Barcode Percentile Cutoff ⓘ

99

Advanced pigeon filter options ⓘ

Advanced pigeon make-seurat options ⓘ

Add task memory (MB) ⓘ

40960

Compute Settings ⓘ

compute9 ⇅

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## Memory needs for single-cell Iso-Seq on large datasets

Certain steps in the Single-Cell Iso-Seq workflow have larger memory requirements. For example, a 300 million S-read (consisting of two Revio<sup>®</sup> SMRT<sup>®</sup> Cell worth of Kinnex single-cell data) Single-Cell Iso-Seq workflow can have a peak memory use of 102 GB and take 27 hours to complete on a typical HPC cluster.

The table below shows the runtime and peak memory usage for some of the key tasks in the Single-Cell Iso-Seq workflow. The memory consumption will be estimated when task jobs are submitted to the cluster, but for very large or pathological datasets it may be necessary to allocate additional memory using the "Add task memory (MB)" options.

Command	Run Time	Peak RSS	Purpose	
isoseq tag	2 hr	0.2 GB	Extract UMI and cell barcodes	
isoseq correct	2.5 hr	73 GB	Match extracted cell barcodes with whitelist and correction	
isoseq groupdedup	7 hr	11 GB	UMI deduplication	
pbbmm2 align	3 hr	42 GB	Align dedup reads to ref genome	
isoseq collapse	2 hr	41 GB	Collapse redundant isoforms	
pigeon classify	33 min	46 GB	Classify isoforms against Gencode annotation	
pigeon filter	17 min	21 GB	Filter cDNA artifacts	
pigeon make-seurat	1.5 hr	102 GB	Make gene- and isoform-level count matrix	
pigeon report	20 min	15 GB	Generate pigeon reports	

Other reasons for why a single-cell Iso-Seq job might have high memory demands or long run times include a large number of cells called (e.g., >100k), a high number of reads concentrated in a small number of cells, high UMI duplicate rate, or low transcript complexity (e.g., targeted enrichment or tissue-specific highly expressed genes).

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## Other useful community tools and tutorials

- (1) Community tutorials - Broad Institute Kinnex bulk and single-cell best practices
- (2) Bulk and single-cell Iso-Seq command line resources: <https://isoseq.how/>
- (3) [Application note - Bioinformatics tools for full-length isoform sequencing](#)

Note: For understanding how pigeon classification and filtering works, see sections “Transcript Mapping and Classification” and Appendix in [SMRT Link Kinnex full-length RNA troubleshooting guide](#)