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Using the Iso-Seq Application on SMRT Link and BioConda

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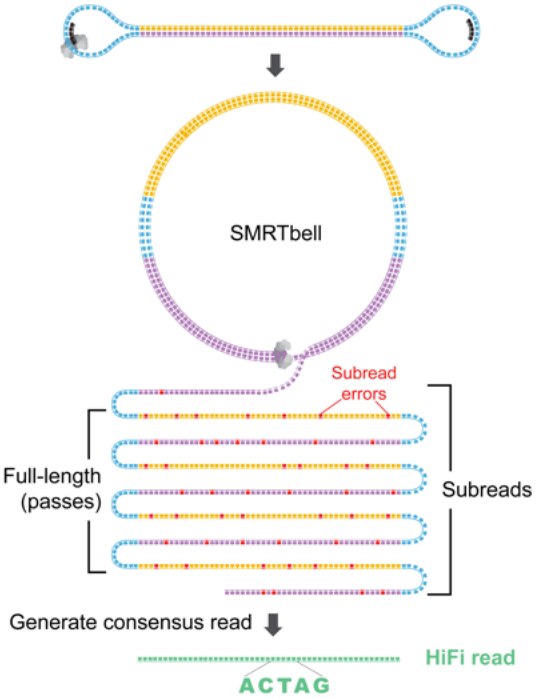
Why use Iso-Seq analysis?

ISO-SEQ ANALYSIS MAIN FEATURES

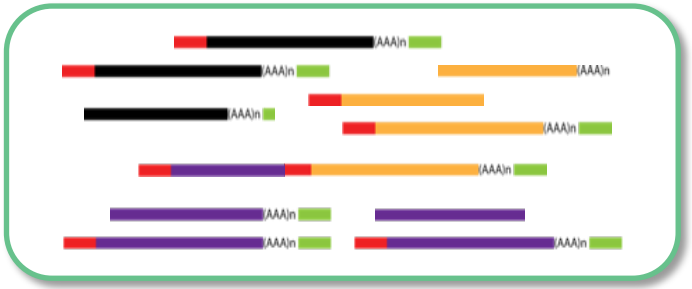
- No reference genome required
- No transcriptome assembly required
- Recovers full-length (5’ to 3’) transcripts
- Yields highly accurate (>99%) transcripts

HIFI READS FROM CCS

CCS → REMOVE PRIMERS → REFINE → CLUSTER → MAP & COLLAPSE



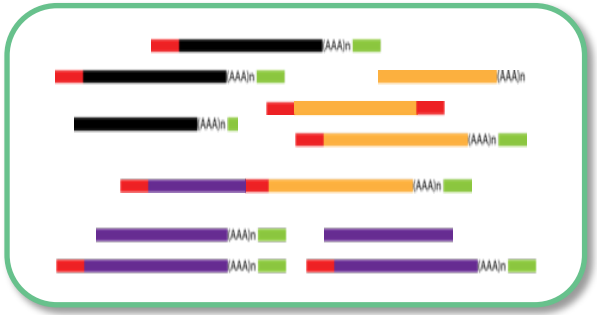
HiFi reads



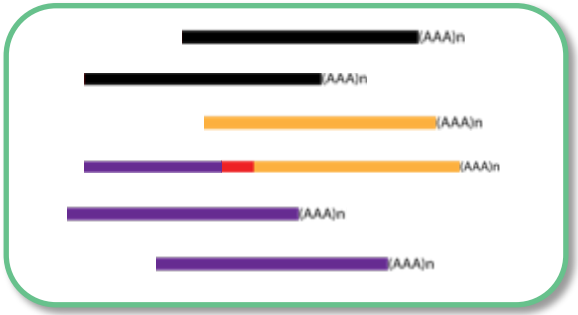
FULL-LENGTH READS HAVE 5’ AND 3’ PRIMERS

CCS → **REMOVE PRIMERS** → REFINE → CLUSTER → MAP & COLLAPSE

HiFi reads



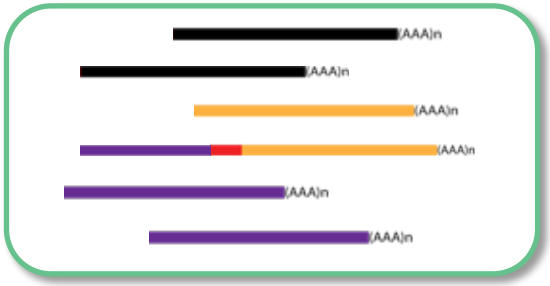
FL reads



REMOVE CONCATEMERS AND POLY(A) TAILS

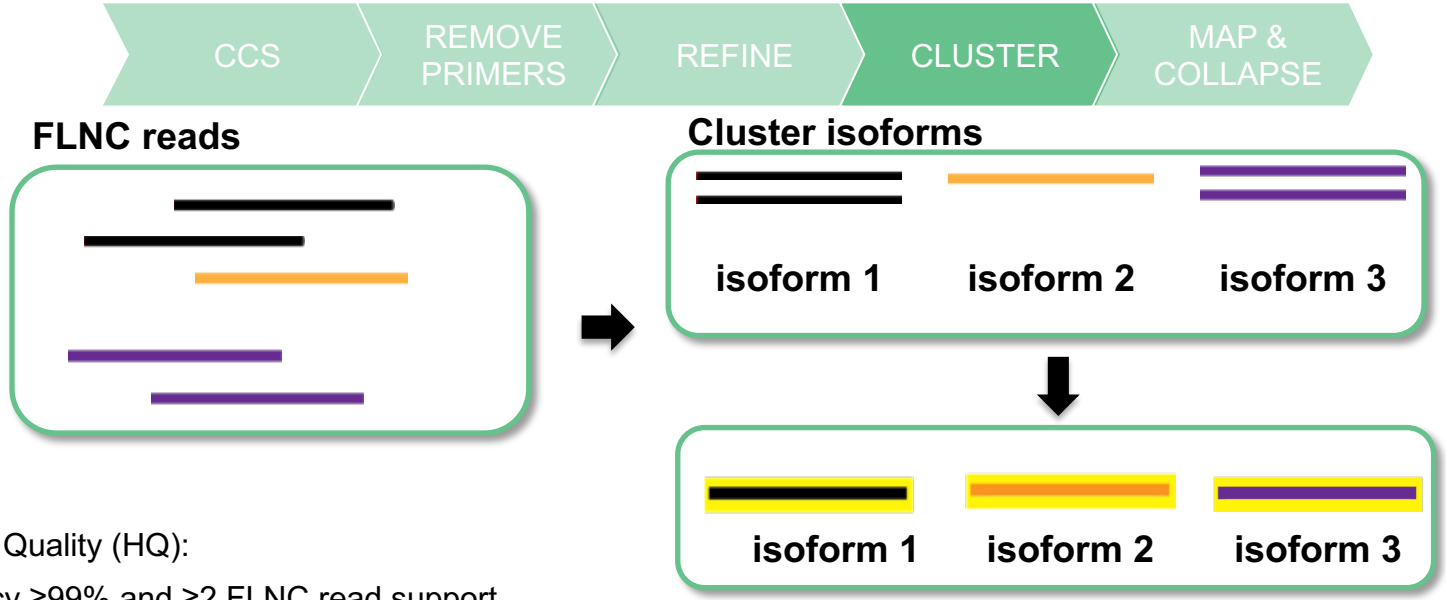
CCS | REMOVE PRIMERS | **REFINE** | CLUSTER | MAP & COLLAPSE

FL reads



FLNC reads

CLUSTER TO GET ISOFORMS



- High Quality (HQ):

accuracy ≥99% and ≥2 FLNC read support

- Low Quality (LQ):

accuracy <99% and ≥2 FLNC read support

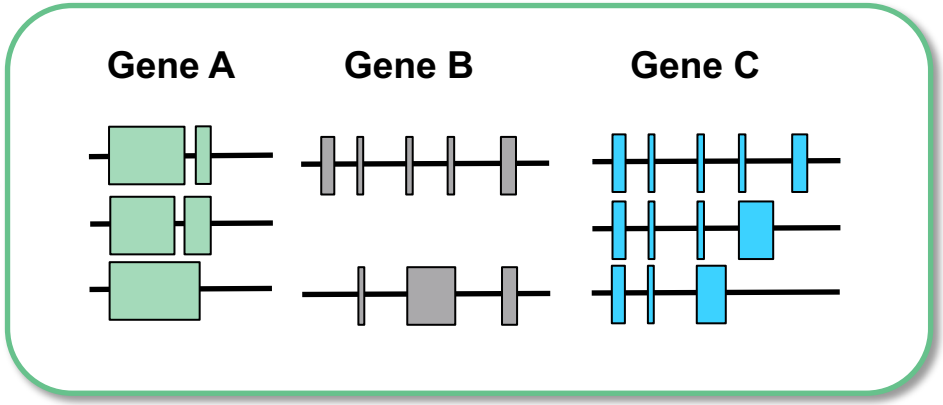
MAP AND COLLAPSE ISOFORMS

CCS → REMOVE PRIMERS → REFINE → CLUSTER → MAP & COLLAPSE

High-Quality, Full-Length Polished Isoforms

Map to Reference Genome

Minimap2 (pbmm2)



BENEFITS OF ISO-SEQ ANALYSIS APPLICATION

- High-quality transcripts
- Full-Length Non-concatemer reads
- Mapped & collapsed isoforms
- Removes artifacts
- Removes poly(A) tails

Iso-Seq Analysis Using pbBioConda

INSTRUCTIONS TUTORIAL

Follow the instructions tutorial for installing all the software needed.

- If you do not have an HPC server to install pbbioconda, you should have already:
  - Create an AWS account
  - Create an AWS Linux Instance to run Iso-Seq 3 Analysis Pipeline
  - Connect to your AWS Instance
- Upgrades and Install Software

DOWNLOAD THE DATA

https://downloads.pacbcloud.com/public/dataset/ISMB_workshop/

Index of /public/dataset/ISMB_workshop/isoseq3

Name	Last modified	Size	Description
Parent Directory		-	
results/	2020-09-23 07:31	-	
alz.ccs.bam	2020-06-15 11:52	84M	
isoseq_primers.fasta	2020-09-23 07:03	62	
run.sh	2020-09-23 07:23	430	

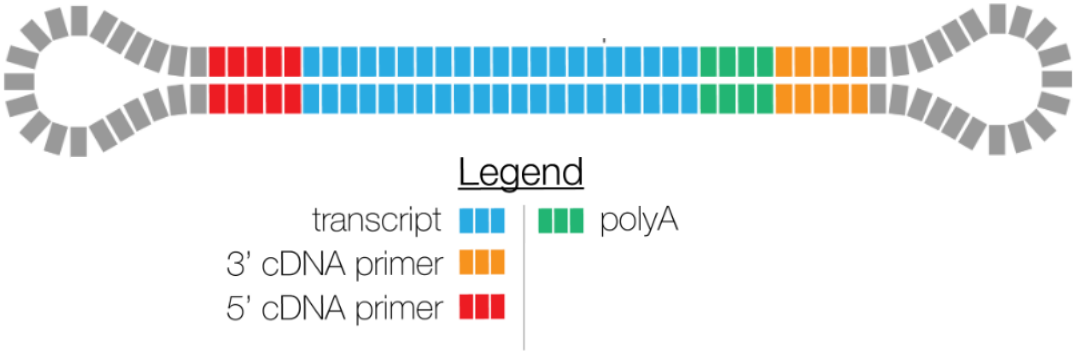
Example:

```
$ wget -nv https://downloads.pacbcloud.com/public/dataset/ISMB_workshop/isoseq3/alz.ccs.bam
```

SPECIFY ISO-SEQ PRIMERS

```
$ more primers.fasta

>5p
GCAATGAAGTCGCAGGGTTGGG
>3p
GTACTCTGCGTTGATACCACTGCTT
```



INPUT CCS BAM FILE

```
$ samtools view -h alz.ccs.bam
```

```
m141008_060349_42194_c100704972550000001823137703241586_s1_p0/63/ccs4*0255
**00CCCGGGGATCCTCTAGAATGC~~~~~~~~~~~~~~~~~~~~~~RG:Z:83ba013f np:i:35
rq:f:0.999682 sn:B:f,11.3175,6.64119,11.6261,14.5199 zm:i:63
```

REFERENCE GENOME

```
$ grep '>’ hg38.fa # to list the headers per chromosome
```

```
>chr1 AC:CM000663.2 gi:568336023 LN:248956422 rl:Chromosome M5:6aef897c3d6ff0c78a
ff06ac189178dd AS:GRCh38
>chr2 AC:CM000664.2 gi:568336022 LN:242193529 rl:Chromosome M5:f98db672eb0993dcfd
abafe2a882905c AS:GRCh38
>chr3 AC:CM000665.2 gi:568336021 LN:198295559 rl:Chromosome M5:76635a41ea913a405d
ed820447d067b0 AS:GRCh38
>chr4 AC:CM000666.2 gi:568336020 LN:190214555 rl:Chromosome M5:3210fecf1eb92d5489
da4346b3fddc6e AS:GRCh38
>chr5 AC:CM000667.2 gi:568336019 LN:181538259 rl:Chromosome M5:a811b3dc9fe66af729
dc0dddf7fa4f13 AS:GRCh38 hm:47309185-49591369
…
```

SOFTWARE INSTALLATION CHECK

Access to your conda environment

```
$ source activate <name of your environment>
```

Check your installation

```
$ isoseq3 --version
isoseq3 3.4.x
$ lima --version
lima 1.11.0
$ pbmm2 --version
pbmm2 1.3.0
```

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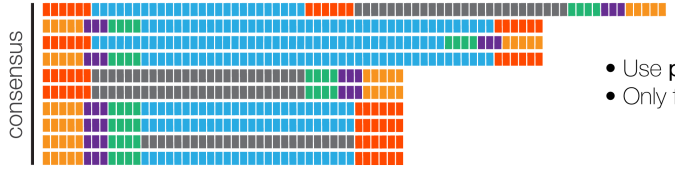
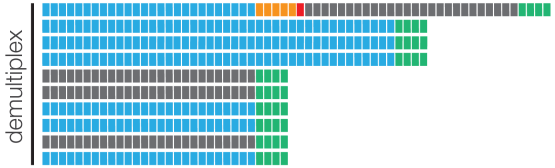
ISO-SEQ WORKFLOW

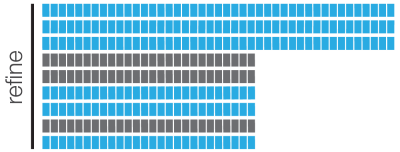
ISO-SEQ WORKFLOW



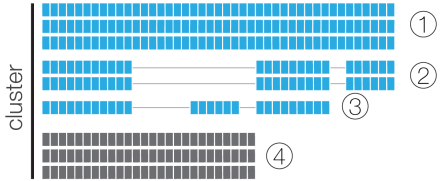
- Use **polished** CCS reads
- Only full-pass ZMWs



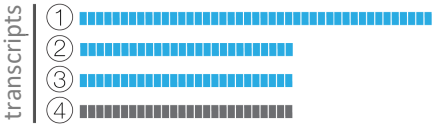
- Barcoded and unbarcoded cDNA primer removal
- Orientation
- Unwanted primer combination removal



- PolyA tail trimming
- Concatemer removal

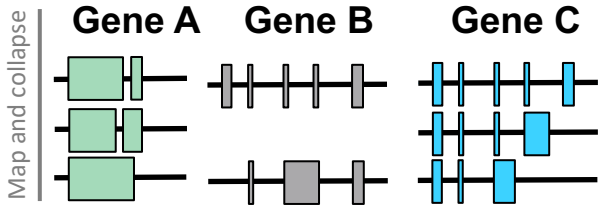


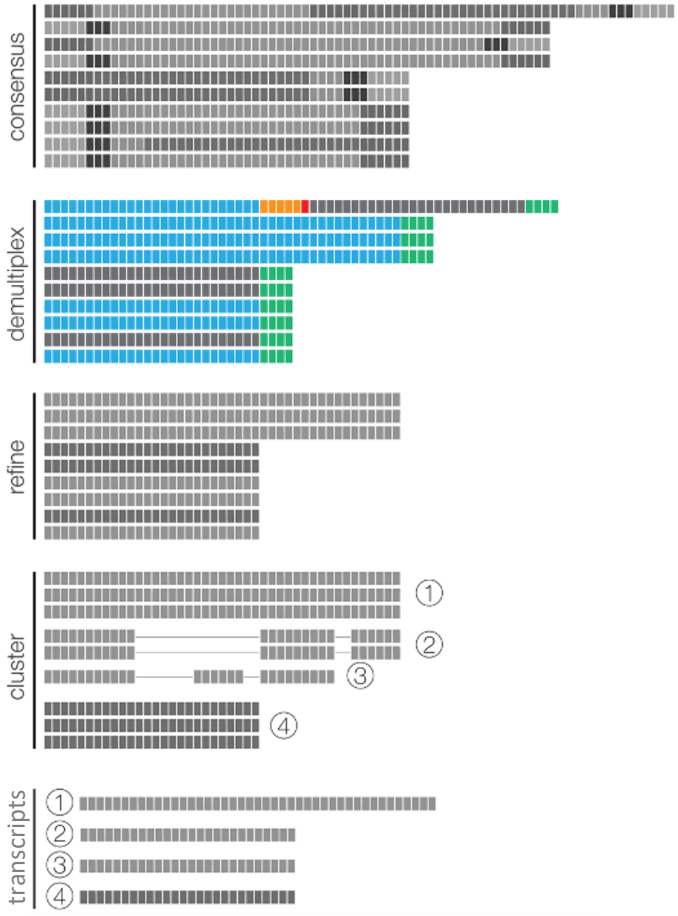
- Hierarchical, n*log(n) clustering, alignment of shorter to longer sequences
- Iterative cluster merging
- Generate consensus for each read cluster using QV guided PoA



- One consensus per read cluster

- Align to reference genome
- Remove redundancy





PRIMER REMOVAL & DEMULTIPLEXING

Command line:

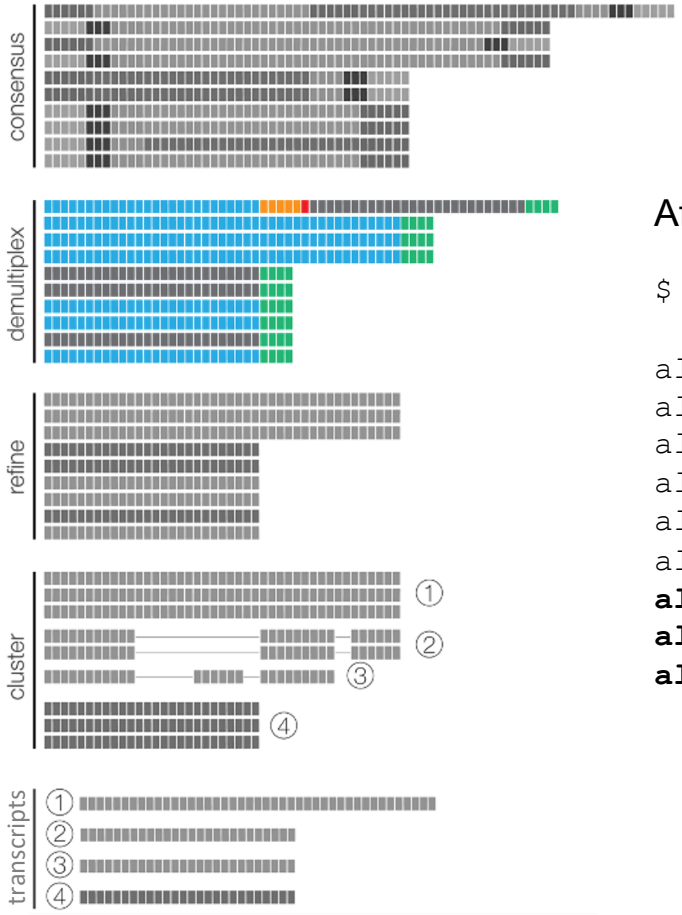
```
lima --isoseq --dump-clips --peek-guess -j 24\
alz.ccs.bam isoseq_primers.fasta alz.demult.bam
```

```
Input files:
     alz.ccs.bam  #HiFi reads
     isoseq_primers.fasta #Iso-Seq primers

Output files:
     alz.demult.bam

Options:
     --isoseq #specialized isoseq option for lima
     --dump-clips # show the clipped primers
     --peek-guess # remove spurious false positive signal
     -j 24 # Number of threads to use
```

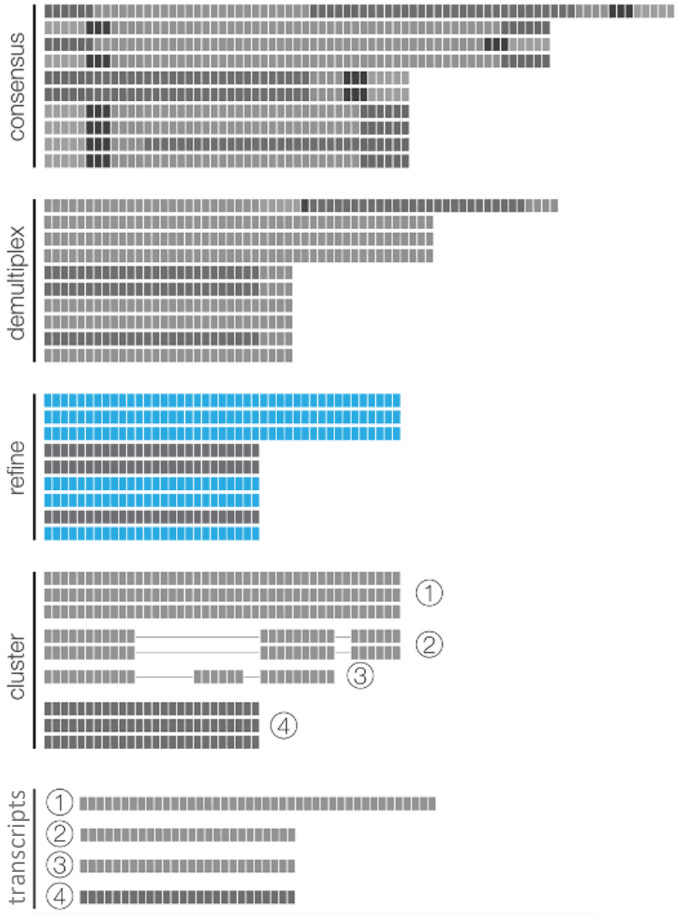
PRIMER REMOVAL & DEMULTIPLEXING



After completion, you will see the following files:

```
$ ls -ltrh

alz.demult.json
alz.demult.lima.clips
alz.demult.lima.counts
alz.demult.lima.guess        #lima reports
alz.demult.lima.report
alz.demult.lima.summary
alz.demult.5p--3p.bam
alz.demult.5p--3p.bam.pbi
alz.demult.5p--3p.subreadset.xml
```



TRIMMING POLY(A) TAILS AND CONCATEMER REMOVAL

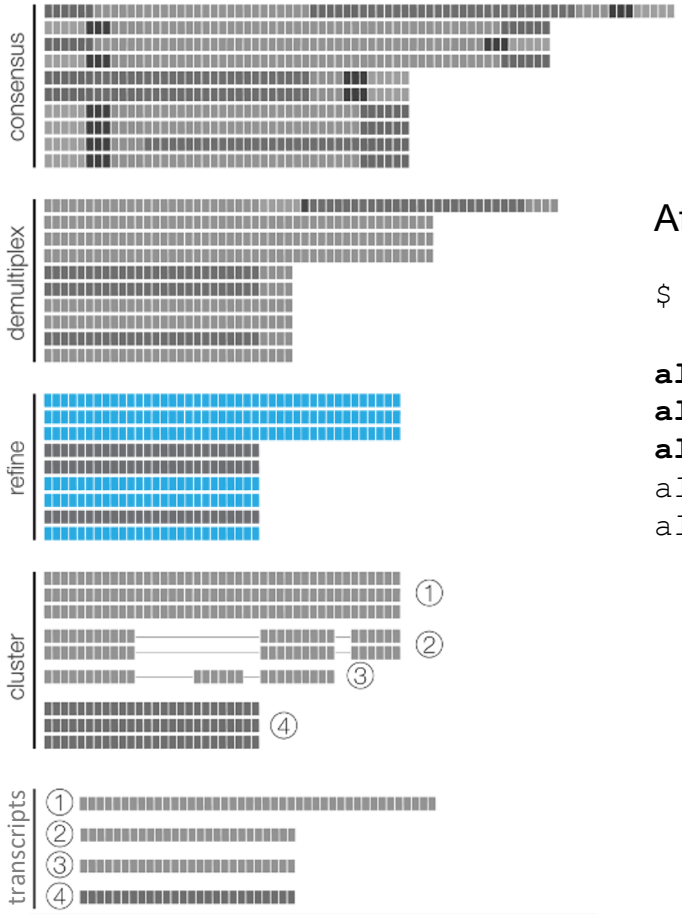
Command line:

```
isoseq3 refine --require-polya\
alz.demult.5p--3p.bam\ isoseq_primers.fasta
alz.flnc.bam
```

```
Input files:
      alz.demult.5p--3p.bam
      isoseq_primers.fasta

Output files:
      alz.flnc.bam

Options:
       --require-polya #if your transcripts have a polyA tail
```



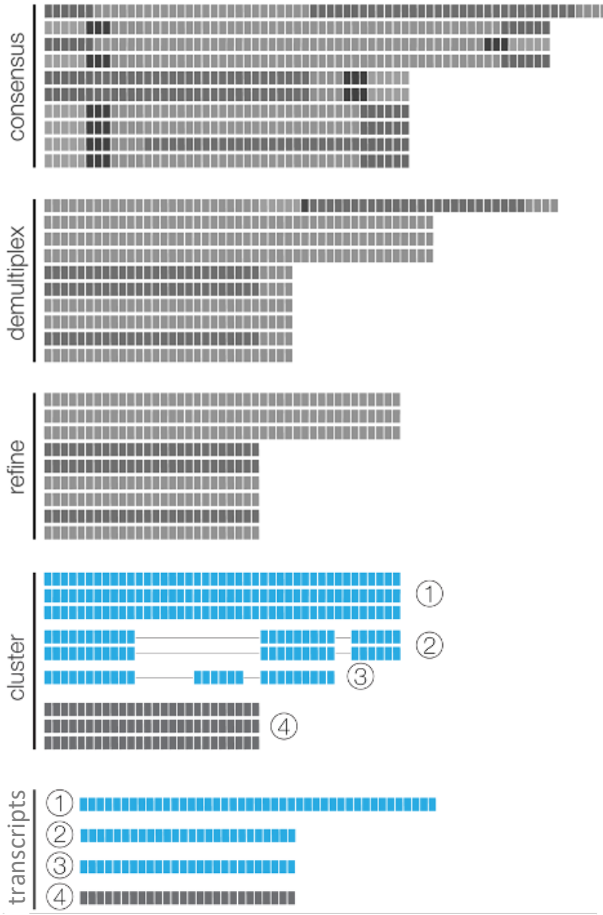
TRIMMING POLY(A) TAILS AND CONCATEMER REMOVAL

After completion, you will see the following files:

```
$ ls -ltrh

alz.flnc.bam
alz.flnc.bam.pbi
alz.flnc.consensusreadset.xml
alz.flnc.filter_summary.json    #isoseq3 refine reports
alz.flnc.report.csv
```

ISOFORMS



Command line:

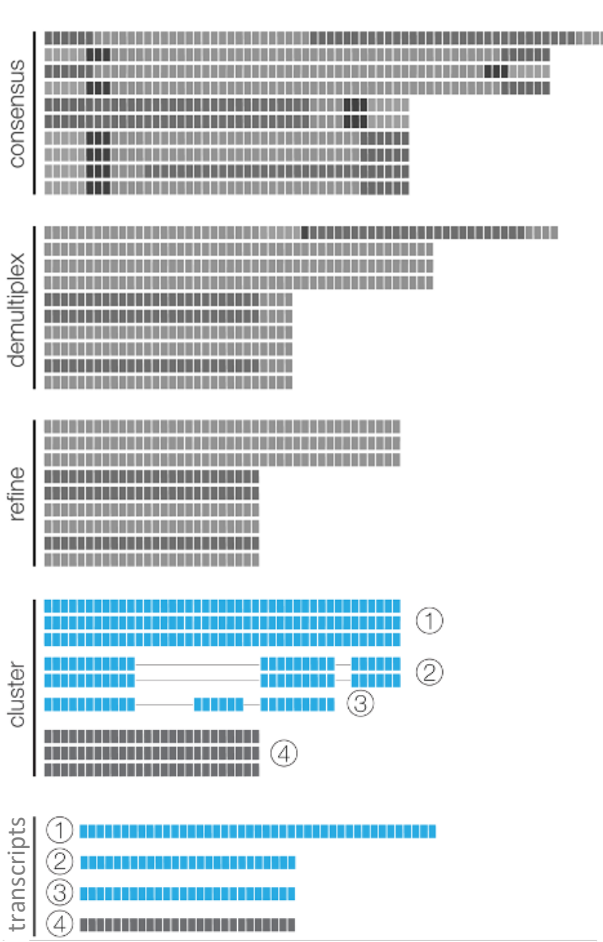
```
isoseq3 cluster alz.flnc.bam alz.polished.bam \
--verbose --use-qvs
```

```
Input files:
      alz.flnc.bam

Output files:
     alz.flnc.bam

Options:
     --verbose #if your transcripts have a polyA tail
     --use-qvs #Use CCS QVs, sets --poa-cov 100
```

ISOFORMS



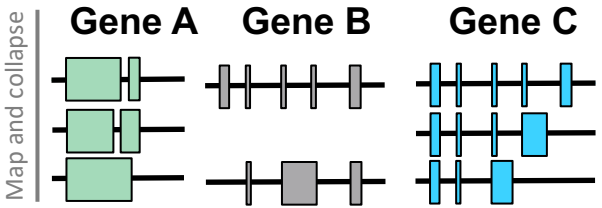
After completion, you will see the following files:

```
$ ls -ltrh

alz.polished.bam
alz.polished.bam.pbi
alz.polished.transcriptset.xml
alz.polished.cluster                  ] #isoseq3 cluster reports
alz.polished.cluster_report.csv       ]
alz.polished.hq.bam                   ]
alz.polished.hq.bam.pbi               ] #high quality isoforms(≥0.99)
alz.polished.hq.fasta.gz              ]
alz.polished.lq.bam                   ]
alz.polished.lq.bam.pbi               ] #low quality isoforms(<0.99)
alz.polished.lq.fasta.gz              ]
```

Because the ccs input is Polished, the isoseq3 **cluster output is already polished!**

MAP



Command line:

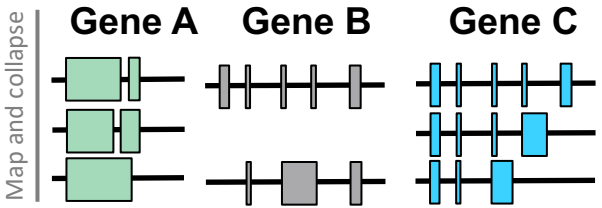
```
pbmm2 align hg38.fa alz.polished.hq.bam
alz.aligned.bam
-j 24 --preset ISOSEQ -sort --log-level INFO
```

```
Input files:
      alz.polished.hq.bam
      hg38.fa

Output files:
       alz.aligned.bam

Options:
      -j 24 #Number of threads to use
      --preset ISOSEQ #select the alignment mode
      --sort #Generate sorted BAM file
      --log-level INFO #show progress
```

MAP

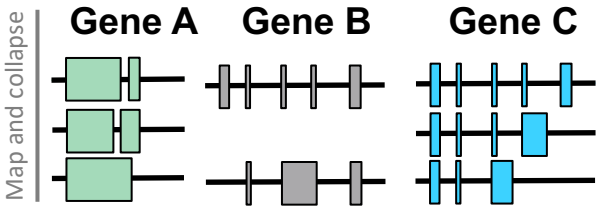


After completion, you will see the following files:

```
$ ls -ltrh

alz.aligned.bam
alz.aligned.bam.bai
```

COLLAPSE



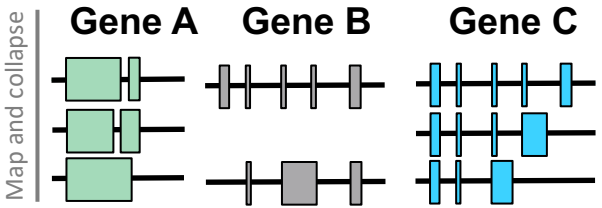
Command line:

```
isoseq3 collapse alz.aligned.bam alz.collapsed.gff
```

Input files:
alz.aligned.bam

Output files:
alz.collapsed.gff

COLLAPSE



After completion, you will see the following files:

```
$ ls -ltrh

alz.collapsed.report.json
alz.collapsed.abundance.txt      #report, stats and list
alz.collapsed.read_stat.txt
alz.collapsed.group.txt
alz.collapsed.gff
alz.collapsed.fasta
```

PUBLICLY AVAILABLE ISO-SEQ DATA SETS

https://github.com/PacificBiosciences/IsoSeq_SA3nUP/wiki/Iso-Seq-in-house-datasets

Iso Seq in house datasets

Elizabeth Tseng edited this page on Oct 8, 2019 · 12 revisions

Last Updated: 10/07/2019

Below are datasets that have been run in-house at PacBio.

Alzheimer Brain (released Oct 2019)

- Download Link

Library prep Iso-Seq® Express Template Preparation for Sequel® and Sequel® II Systems

Sequencing Sequel II System with Sequel II Binding Kit 1.0 and Sequel II Sequencing Kit 1.0

Run time: 24 hrs pre-extension, 4 movie time. Sequenced on 1 SMRT Cell 8M.

ISO-SEQ ANALYSIS TERMINOLOGY

NAME	ABBR	EXPLANATION
Full-Length Reads	FL Reads	CCS reads with 5’ and 3’ cDNA primers removed
Full-Length, Non-Concatemer Reads	FLNC Reads	CCS reads with 5’ and 3’ cDNA primers, polyA tail, and concatemers removed
High-Quality Isoforms	HQ Isoforms	Polished transcript sequences with predicted accuracy ≥99% & ≥2 FLNC
Low-Quality Isoforms	LQ Isoforms	Polished transcript sequences with predicted accuracy <99% & ≥2 FLNC

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