Version 7

Oct 30, 2022

Preparing Reads for Stranded Mapping V.7

In 5 collections

David A Eccles[1]

[1]Malaghan Institute of Medical Research (NZ)

1 *Works for me* Share

dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7

David A Eccles
Malaghan Institute of Medical Research (NZ)

ABSTRACT

This protocol is for preparing long reads for stranded mapping, as an intermediate step for additional protocols:

- Aligning strand-oriented sequences to a transcriptome for transcript / gene counting
- Aligning strand-oriented sequences to a genome for confirmatory QC

Input(s): demultiplexed fastq files (see protocol Demultiplexing Nanopore reads with LAST), adapter file (containing strand-sensitive adapter sequences)

Output(s): oriented read files, as gzipped fastq files

DOI

dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7

PROTOCOL CITATION

David A Eccles 2022. Preparing Reads for Stranded Mapping. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7
Version created by David A Eccles

COLLECTIONS

Nanopore Data Analysis

Nanopore Data Analysis

Nanopore Data Analysis

Nanopore Data Analysis

Nanopore Data Analysis

KEYWORDS

long reads, nanopore, strand-specific, sequencing, RNASeq

CREATED

Oct 30, 2022

LAST MODIFIED

Oct 30, 2022

PROTOCOL INTEGER ID

72044

PARENT PROTOCOLS

Part of collection

Nanopore Data Analysis
Nanopore Data Analysis
Nanopore Data Analysis
Nanopore Data Analysis
Nanopore Data Analysis

Barcode Demultiplexing

1 Demultiplex reads as per protocol Demultiplexing Nanopore reads with LAST.

I usually inspect the barcode_counts.txt file, and delete any barcodes that I'm not interested in:

```
cp barcode_counts.txt barcode_counts.orig.txt
nano barcode_counts.txt
```

If demultiplexing has been done correctly, then the following command should produce output without errors:

```
for bc in $(awk '{print $2}' barcode_counts.txt);
  do ls demultiplexed/reads_${bc}.fq.gz;
done
```

Example output:

```
demultiplexed/reads_BC03.fq.gz
demultiplexed/reads_BC04.fq.gz
demultiplexed/reads_BC05.fq.gz
demultiplexed/reads_BC06.fq.gz
```


Citation: David A Eccles Preparing Reads for Stranded Mapping https://dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7

```
demultiplexed/reads_BC07.fq.gz
demultiplexed/reads_BC08.fq.gz
```

If the *barcode_counts.txt* file is missing, the output will look like this:

```
awk: fatal: cannot open file `barcode_counts.txt' for reading (No
such file or directory)
```

If one or more of the barcode-demultiplexed files are missing, the output will look something like this:

```
demultiplexed/reads_BC03.fq.gz
demultiplexed/reads_BC04.fq.gz
demultiplexed/reads_BC05.fq.gz
ls: cannot access 'demultiplexed/reads_BC06.fq.gz': No such file or
directory
ls: cannot access 'demultiplexed/reads_BC07.fq.gz': No such file or
directory
demultiplexed/reads_BC08.fq.gz
```

Index Preparation

2 Prepare and index a FASTA file containing adapter sequences (see attached FASTA file).

adapter_seqs.fa

Create a shell variable containing this file name:

```
adapterFile="adapter_seqs.fa"
```

3 Prepare the LAST index for the adapter file. Newer versions of LAST (v1409+) include a new seeding scheme, '-uRY4' [and other related RYX schemes], which improves mapping accuracy and reduces polyA matches; low-complexity regions are also converted to lower case with '-R01'. This will generate seven additional files of the form <index name>.XXX:

```
lastdb -uRY4 -R01 ${adapterFile} ${adapterFile}
```

4 Prepare a substitution matrix for adapter mapping. Mapping seems to work better when Q values are included:

```
#last -Q 1
#last -t4.49549
#last -a 46
#last -A 46
#last -b 3
```


Citation: David A Eccles Preparing Reads for Stranded Mapping https://dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7

```
#last -B 3
#last -S 1
# score matrix (query letters = columns, reference letters = rows):
       A      C      G      T
A      6    -34    -33    -35
C    -34      6    -33    -34
G    -33    -33      7    -34
T    -35    -34    -34      6
```

adapter.mat

A matrix like this can be generated by the following command, which runs *last-train* on the first 10,000 reads from the full read dataset:

```
last-train -Q 1 -P 10 ${adapterFile} <(zcat reads_all.fastq.gz |
head -n 40000) | tail -n 13
```

[note: it is also fine to use the same matrix as used for demultiplexing]

Orienting Reads

5 Use LAST in *split* mode, using the pre-defined substitution matrix to map the reads. In this example, it is distributed over 10 processing threads (-P 10). In this case it's important that the direction of mapping is also recorded, so the *cut* command selects three fields (query name [7], target name [2], mapping direction [10]):

```
for bc in $(awk '{print $2}' barcode_counts.txt);
  do echo "** ${bc} **";
  lastal --split -P10 -p adapter.mat ${adapterFile} <(pv
demultiplexed/reads_${bc}.fq.gz) | \
    maf-convert -n tab | cut -f 2,7,10 | uniq | \
    gzip > demultiplexed/adapter_assignments_${bc}.txt.gz
done
```

6 The adapter assignments are filtered through *uniq* in order to catch (and exclude) any reads with the strand-switch primer matching multiple times. To unpack the *uniq* pipe a little bit more, it skips the first field (adapter name), then matches up to 36 characters, retaining only lines that don't match any others. This catches a few more chimeric reads that were missed by the unique barcode filter in the previous protocol.

Reads are filtered into two groups (and one group-by-omission) based on the mapped direction of the strand-switch primer, then reverse-complemented (if necessary) to match the orientation of the original RNA strand. I use my fastx-fetch.pl and fastx-rc.pl scripts for this.

Citation: David A Eccles Preparing Reads for Stranded Mapping https://dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7

fastx-fetch.pl

fastx-rc.pl

```
mkdir -p oriented
for bc in $(awk '{print $2}' barcode_counts.txt);
 do echo "** ${bc} **";
 fastx-fetch.pl -i <(zgrep '^SSP'
demultiplexed/adapter_assignments_${bc}.txt.gz | \
     sort | uniq -f 1 -w 36 -u | \
     awk '{if($3 == "+"){print $2}}') <(pv
demultiplexed/reads_${bc}.fq.gz) | \
   gzip > oriented/${bc}_reads_fwd.fq.gz
 fastx-fetch.pl -i <(zgrep '^SSP'
demultiplexed/adapter_assignments_${bc}.txt.gz | \
     sort | uniq -f 1 -w 36 -u | \
     awk '{if($3 == "-"){print $2}}') <(pv
demultiplexed/reads_${bc}.fq.gz) | \
   fastx-rc.pl | gzip > oriented/${bc}_reads_rev.fq.gz
done
```

7 Forward and reverse-oriented sequences are combined together to form a single group of RNA-oriented reads.

```
for bc in $(awk '{print $2}' barcode_counts.txt);
  do echo "** ${bc} **";
  pv oriented/${bc}_reads_fwd.fq.gz oriented/${bc}_reads_rev.fq.gz
| \
    zcat | gzip > oriented/${bc}_reads_dirAdjusted.fq.gz
done
```

Downstream Workflows

8 Following on from here, the oriented reads can be mapped to a genome (e.g. for visual confirmation of mapping), or to a transcriptome (e.g. for read counting):

- Stranded Mapping from Oriented Long Reads
- Stranded Transcript Count Table Generation from Long Reads

Citation: David A Eccles Preparing Reads for Stranded Mapping https://dx.doi.org/10.17504/protocols.io.5qpvon2zzl4o/v7