Plasmid Sequence Analysis from Long Reads V.5

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1 Works for me
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dx.doi.org/10.17504/protocols.io.bwj6pcre

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ABSTRACT

This protocol demonstrates how to assemble reads from plasmid DNA, and generate a circularised and non-repetitive consensus sequence

At the moment, this protocol uses Canu to de-novo assemble high-quality single-cut reads.

Input(s):

- demultiplexed fastq files (see protocol Demultiplexing Nanopore reads with LAST). I've noticed that the default demultiplexing carried out by Guppy (at least up to v4.2.2, as used in the first version of this protocol) has issues with chimeric reads, which can affect assembly.

Output(s):

- Consensus sequence per barcode as a fasta file

DOI

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PROTOCOL CITATION

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Read file preparation

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

If this has been done, 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_BC02.fq.gz
demultiplexed/reads_BC03.fq.gz
demultiplexed/reads_BC04.fq.gz
demultiplexed/reads_BC05.fq.gz
demultiplexed/reads_BC07.fq.gz
demultiplexed/reads_BC09.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_BC02.fq.gz
demultiplexed/reads_BC03.fq.gz
demultiplexed/reads_BC04.fq.gz
ls: cannot access 'demultiplexed/reads_BC05.fq.gz': No such file or directory
ls: cannot access 'demultiplexed/reads_BC07.fq.gz': No such file or directory
demultiplexed/reads_BC09.fq.gz
```

1.1 If reads have been demultiplexed by MinKNOW, then the following approach should work to create the right input format:

```
mkdir demultiplexed;
# readlocation is the directory that contains barcode subdirectories
readLocation = "../*/fastq_pass"; # or "../*/pass" for Guppy
for x in $(ls ${readLocation}); do
  echo ${x};
  cat ${readLocation}/${x}/*.fastq | gzip > demultiplexed/reads_${x}.fq.gz;
  echo "1 ${x}" >> barcode_counts.txt;
done
```

2 Create a directory to store results files

```
mkdir results
```

3 Determine the N50/L50 read length for each barcode. This will be used as the initial guess at the assembly size.

```
(for bc in $(awk '{print $2}' barcode_counts.txt);
    do echo -n ${bc};
    fastx-length.pl demultiplexed/reads_${bc}.fq.gz 2>&1 > /dev/null | \
      grep L50 | awk '{print "\t"$5$6}' | perl -pe 's/b$//';
 done) > results/read_L50.txt
```

This file can be viewed to confirm the assembly lengths:

```
cat results/read_L50.txt
```

Example output:

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```
BC02    347
BC03    8.904k
BC04    8.888k
BC05    10.262k
BC07    11.076k
BC09    11.093k
```

Note: this file will be used for subsequent downstream processing. If any of these barcodes shouldn't be processed further, feel free to remove the corresponding lines from this file

Read filitering

4 Filter out any reads that are less than half of the target read length, and determine the average quality of the remainder, keeping information on (at most) 100 of the highest-quality reads:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  ~/scripts/fastx-compstats.pl demultiplexed/reads_${bc}.fq.gz | \
    sort -t ',' -k 16rn,16 | \
    awk -F ',' -v "len=${len}" \
      'BEGIN{if(match(len, "k") > 0){sub("k","",len); len=len*1000}}
       {if($18 > (len / 2)){print}}' | \
    head -n 100 | grep -v '^name' > results/bestLong_100X_${bc}.csv;
done
```

Check how successful the sequencer was at getting 100X coverage by counting lines:

```
wc -l results/bestLong_100X_*.csv
```

Example output. In this case BC02 has fewer than 100 reads, so the assembly is less likely to be high-quality:

```
 90 results/bestLong_100X_BC02.csv
100 results/bestLong_100X_BC03.csv
100 results/bestLong_100X_BC04.csv
100 results/bestLong_100X_BC05.csv
100 results/bestLong_100X_BC07.csv
100 results/bestLong_100X_BC09.csv
590 total
```

5 Subset the original read sets to only include the high-quality long reads:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  ~/scripts/fastx-fetch.pl -i results/bestLong_100X_${bc}.csv \
    demultiplexed/reads_${bc}.fq.gz > results/bestLong_100X_${bc}.fastq; done
```

Choice 1: mapping to a reference sequence

6 Create a LAST index for the reference sequence:

```
lastdb -uNEAR -R01 reference/refName.fa reference/refName.fa
```

7 Prepare a substitution matrix for barcode mapping. The default substitution matrix is swayed too much by INDELs in

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the barcode sequences, so here's one that I've developed using a combination of trial & error and last-train. This should work at least for reads called with Guppy v4.2.2:

```
#last -Q 0
#last -t4.385
#last -a 16
#last -A 19
#last -b 4
#last -B 3
#last -S 1
# score matrix (query letters = columns, reference letters = rows):
       A      C      G      T
A      7    -26    -10    -24
C    -26      5    -32    -22
G    -10    -32      5    -29
T    -24    -22    -29      7
```

plasmid_best100.mat

This matrix has a moderate penalty for opening gaps (i.e. insertions and deletions), and a lower penalty for inserting them. Insertions are slightly less likely than deletions. It also has a moderate penalty for A/G transition variants, and a higher penalty for C/T transition variants (but still lower than other substitution penalties).

If you're interested in training your own model based on this one, you can use *last-train* as follows:

```
last-train --matsym -Q 0 -p plasmid_best100.mat \
  reference/refName.fa results/bestLong_100X_BC*.fastq
```

Copy the last few lines from the output into a text file (e.g. `*plasmid_better.mat*`)

8 Map reads to the reference and convert to BAM format using *maf-convert* and *samtools*:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  lastal -j 7 -Q 0 -p plasmid_best100.mat reference/refName.fa \
      results/bestLong_100X_${bc}.fastq | last-split -n -m 0.99 | last-postmask | \
    maf-convert sam | samtools view -h --reference reference/refName.fa | \
    samtools sort > results/${bc}_vs_refName.bam
  samtools index results/${bc}_vs_refName.bam
done
```

9 Split reads into forward and reverse-mapped sequences, and tally up base counts at each location:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  samtools view -b -F 0x10 results/${bc}_vs_refName.bam | \
    samtools mpileup --reference reference/refName.fa -B -Q 0 - | \
    ~/scripts/readstomper.pl -c > results/fwd_stomped_${bc}_vs_refName.csv
  samtools view -b -f 0x10 results/${bc}_vs_refName.bam | \
    samtools mpileup --reference reference/refName.fa -B -Q 0 - | \
    ~/scripts/readstomper.pl -c > results/rev_stomped_${bc}_vs_refName.csv
done
```


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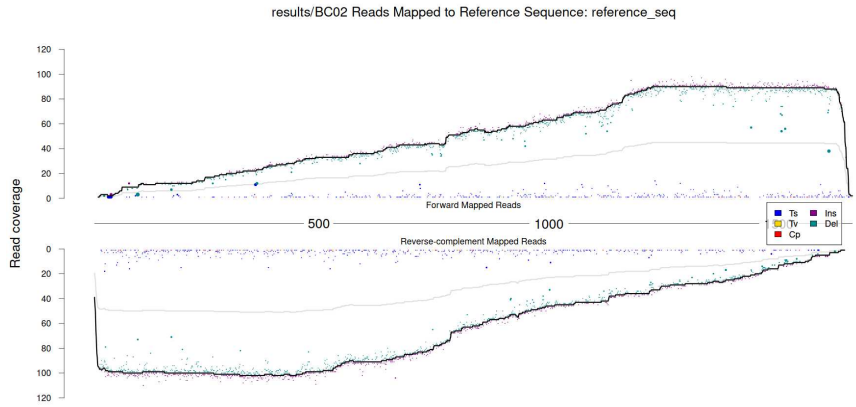
10 Create a visualisation of the base-stomped output:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  ~/scripts/stomp_plotter.r -f results/fwd_stomped_BC02_vs_refName.csv \
    -r results/rev_stomped_BC02_vs_refName.csv -prefix results/BC02
done
```

Example output (`*results/BC02_stompPlot_reference_seq.png*`):



Choice 2: De-novo Canu Assembly

11 Run a Canu assembly on each read set, using default options:

```
cat results/read_L50.txt | while read bc len;
  do canu -nanopore results/bestLong_100X_${bc}.fastq \
    -p ${bc} -d results/canu_${bc} genomeSize=${len};
done
```

12 Trim the assembled contigs based on Canu's trim recommendations:

```
cat results/read_L50.txt | while read bc len;
  do echo ${bc} ${len};
  samtools faidx results/canu_${bc}/${bc}.contigs.fasta \
    $(grep '^>' results/canu_${bc}/${bc}.contigs.fasta | \
      perl -pe 's/^>(.*?) .*trim=(.*)/$1:$2/') > results/circTrimmed_${bc}.fasta;
done
```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