**PIPEBAR and OverlapPER: tools for a fast and accurate DNA barcoding analysis and paired-end assembly**

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# Supplementary material

## Obtaining the test dataset:

The whole dataset used as input to PIPEBAR, SeqTrace and Geneious can be downloaded at https://sourceforge.net/projects/pipebar/files/TraceFiles/

## PIPEBAR automatic installation

To facilitate the use of PIPEBAR by the users, we created a docker image which will enable the user to run PIPEBAR without installing its dependencies.

### **Installation using docker (**see https://docs.docker.com**):**

A docker image is available so the installation of all required tools are already wraped up for usage along PIPEBAR.

**Step 1 – Installing Docker and wget (prerequisites)**

>> sudo apt-get install docker.io

```
renato@ITVDS-WS056: ~
renato@ITVDS-WS056:~$ sudo apt-get install docker.io
```

>> sudo apt-get install wget

```
renato@ITVDS-WS056: ~
renato@ITVDS-WS056:~$ sudo apt-get install wget
```

**Step 2 – Checking Docker installation**

>> sudo docker --version

```
renato@ITVDS-WS056: ~
renato@ITVDS-WS056:~$ sudo docker --version
Docker version 1.13.1, build 092cba3
```

**Step 3 – Downloading the Pipebar Script**

In this step, you will download the script, available on SourceForge, that automatize the Pipebar pipeline. To download the script, enter:

>>wget https://sourceforge.net/projects/pipebar/files/pipebarScript.sh

```
renato@ITVDS-WS056: ~
renato@ITVDS-WS056:~$ wget https://sourceforge.net/projects/pipebar/files/pipeba
rScript.sh
--2018-05-23 15:16:38--  https://sourceforge.net/projects/pipebar/files/pipebarS
cript.sh
Resolvendo sourceforge.net (sourceforge.net)... 216.105.38.13
Conectando-se a sourceforge.net (sourceforge.net)|216.105.38.13|:443... conectad
o.
A requisição HTTP foi enviada, aguardando resposta... 302 Found
Localização: https://sourceforge.net/projects/pipebar/files/pipebarScript.sh/dow
nload [redirecionando]
```

**Step 4 - Initiating Pipebar**

After downloading the script, you will be able to run the pipeline. With superuser permission you will type:

>>sudo sh pipebarScript.sh path/to/forward/reads path/to/reverse/reads
You need to pass two parameters, the path to forward and reverse reads. Once you entered the above command you will get a similar output, regarding the creation of the Pipebar container.

```
root@c54f2d02f1e1: /
renato@ITVDS-WS056:~$ sudo sh pipebarScript.sh Forward/ Reverse/
Creating a Pipebar Container:
c54f2d02f1e1707c751bb10d8f01b74d32b445d87be6b44ede9e04e68ef29433
Running a Pipebar Container:
pipebar
Usage: ./pipebar --format <"ab1", "fastq" or "fastaqual"> --sep <separator_of_fo
rward/reverse_reads> --mo <min_overlap> --ms <min_similarity> --phred <phred_off
set> -q <phred_threshold> --coding <"1" for coding sequences or "0" for non-codi
ng sequence> --gcode <"1" for Standard code, "2" to Vertebrate Mitochondrial Cod
e, "5" to Invertebrate Mitochondrial Code or "11" to Bacterial, Archaeal and Pla
nt plastid code> --rep <"full" or "fast" report>
Default: ./pipebar
Default running includes: --format ab1 --sep _ --mo 25 --ms 0.9 --phred 33 -q 20
 --coding 1 --gcode 1 --rep fast
root@c54f2d02f1e1:/# ./pipebar
```

**Step 5 - Running Pipebar**

At this point you will be enabled to run the pipeline, as it follows.

>./pipebar --format <"ab1", "fastq" or "fastaqual"> --sep <separator_of_forward/reverse_reads> --mo <min_overlap> --ms <min_similarity> --phred <phred_offset> -q <phred_threshold> --coding <"1" for coding sequences or "0" for non-coding" sequence> --gcode <"1" for Standard code, "2" to Vertebrate Mitochondrial Code, "5" to Invertebrate Mitochondrial Code or "11" to Bacterial, Archaeal and Plant plastid code> --rep <"full" or "fast" report>

Ex: ./pipebar --format ab1 --sep _ --mo 25 --ms 0.9 --phred 33 -q 20 --coding 1 --gcode 1 --rep fast

```
root@c54f2d02f1e1:/
root@c54f2d02f1e1:/# ./pipebar
working with forward.fastq
Input and filter stats:
        Input sequences: 436
```

Options:

-h|--help
Show this output.
-V|--version
Show version information.
--format <string>
Input format. Can be "ab1", "fastq" or "fastaqual".
--sep <string>
The IDs from both forward and reverse reads must have a separator.
Ex: 001read_forward and 001read_reverse have "_" (default) as separator
--mo <integer>
Length of the minimum overlap between the paired reads (default is 25).
--ms <float>
Percentage of the accepted minimum similarity in an overlap region of two paired reads (default is 0.9).
--phred <integer>
The offset of the PHRED qualities codes used.
Can be 33 or 64 (default is 33).
-q <integer>
The minimum quality value for trimming and filtering steps (default is 20).
--coding <integer>
Inform if the barcode sequences to be analyzed are from coding (e.g. rbcL, matK) or non-coding (e.g. ITS, atpF-trnH) regions.
Inform "1" for coding or "0" for non-coding sequences (default is 1)
--gcode <integer>
The genetic code to be used when translating the nucleotide sequences into protein, when it comes to a coding region. It can be "1" to Standard Code, "2" to Vertebrate Mitochondrial Code, "5" to Invertebrate Mitochondrial Code or "11" to Bacterial, Archaeal and Plant plastid code.
--rep <string>
A full report will generate a quality graphical report for each barcode sequence analyzed, while a fast report will generate an overview of the analyzed barcodes in one single report (default is "fast")

When the pipeline finishes its execution, you need to exit the pipebar environment, just enter:
>>exit

```
root@38171f254d55:/# exit
exit
Stopping Container:
pipebar
Removing Container:
pipebar
Done!
renato@ITVDS-WS056:~$
```

**Step 6 - Getting the Results**

The pipebar script saves the results in the ResultPipebar folder that is in the same directory from where it was called. The resulting files are:

- notAssembled-1.fastq
- notAssembled-2.fastq
- overlaped.fasta
- overlapped.fastq
- report.pdf
- TrimmedStop_DNA.fasta
- TrimmedStop_Prot.fasta
- fastqc_report
  - overlapped_fastqc.html
  - overlapped_fastqc.zip

```
renato@ITVDS-WS056:~$ ls ResultPipebar/
fastqc_report          overlaped.fasta    TrimmedStop_DNA.fasta
notAssembled-1.fastq   overlapped.fastq   TrimmedStop_Prot.fasta
notAssembled-2.fastq   report.pdf
renato@ITVDS-WS056:~$
```

## PIPEBAR manual installation

## Installing dependencies manually:

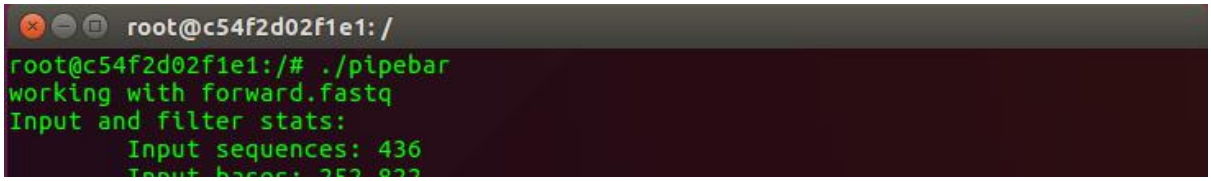
You will need to download the following packages and install them:
- **EMBOSS**: ftp://emboss.open-bio.org/pub/EMBOSS/old/6.5.0/EMBOSS-6.5.7.tar.gz
- **Prinseq**: http://prinseq.sourceforge.net/
- **fastx_toolkit**: http://hannonlab.cshl.edu/fastx_toolkit/
- **BBmap**: https://sourceforge.net/projects/bbmap/?source=typ_redirect
- **FastQC**: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
- **OverlapPER**: https://sourceforge.net/projects/PIPEBAR/files/overlapper.py/download

**Step 1 - Running Pipebar**

At this point you will be enabled to run the pipeline, as it follows.

>./pipebar --format <"ab1", "fastq" or "fastaqual"> --sep <separator_of_forward/reverse_reads> --mo <min_overlap> --ms <min_similarity> --phred <phred_offset> -q <phred_threshold> --coding <"1" for coding sequences or "0" for non-coding" sequence> --gcode <"1" for Standard code, "2" to Vertebrate Mitochondrial Code, "5" to Invertebrate Mitochondrial Code or "11" to Bacterial, Archaeal and Plant plastid code> --rep <"full" or "fast" report>

Ex: ./pipebar --format ab1 --sep _ --mo 25 --ms 0.9 --phred 33 -q 20 --coding 1 --gcode 1 --rep fast




Options:

-h|--help
Show this output.
-V|--version
Show version information.
--format <string>
Input format. Can be "ab1", "fastq" or "fastaqual".
--sep <string>
The IDs from both forward and reverse reads must have a separator.
Ex: 001read_forward and 001read_reverse have "_" (default) as separator
--mo <integer>
Length of the minimum overlap between the paired reads (default is 25).
--ms <float>
Percentage of the accepted minimum similarity in an overlap region of two paired reads (default is 0.9).
--phred <integer>
The offset of the PHRED qualities codes used.
Can be 33 or 64 (default is 33).
-q <integer>
The minimum quality value for trimming and filtering steps (default is 20).
--coding <integer>
Inform if the barcode sequences to be analyzed are from coding (e.g. rbcL, matK) or non-coding (e.g. ITS, atpF-trnH) regions.
Inform "1" for coding or "0" for non-coding sequences (default is 1)
--gcode <integer>
The genetic code to be used when translating the nucleotide sequences into protein, when it comes to a coding region. It can be "1" to Standard Code, "2" to Vertebrate Mitochondrial Code, "5" to Invertebrate Mitochondrial Code or "11" to Bacterial, Archaeal and Plant plastid code.

--rep <string>
A full report will generate a quality graphical report for each barcode sequence analyzed, while a fast report will generate an overview of the analyzed barcodes in one single report (default is "fast")

**Step 2 - Getting the Results**

The resulting files are:

- notAssembled-1.fastq
- notAssembled-2.fastq
- overlaped.fasta
- overlapped.fastq
- report.pdf
- TrimmedStop_DNA.fasta
- TrimmedStop_Prot.fasta
- fastqc_report
  - overlapped_fastqc.html
  - overlapped_fastqc.zip

## OverlapPER tests:

## Simulated Dataset creation:

ART was used to simulate the Illumina sequencing of 1,000,000 paired end reads.

```
>art_illumina -ss MSv3 -p -sam -na -i ecoli_K-12.fasta -l 250 -c 1000000 -ir 0.09 -ir2 0.015 -dr 0.011 -dr2 0.023 -m 400 -s 10 -o illumina_ecoli_ART
```

The simulated data is available on https://sourceforge.net/projects/overlapper-reads/

## Tools commands

**OverlapPER:**

```
>time python3 overlapper.py -f  illumina_ecoli_ART1.fq -r illumina_ecoli_ART2.fq --mo 10 --ms 0.9
```

Merged reads: 999706

Not merged reads: 294

```
real    8m38.279s
user    8m30.844s
sys     0m7.340s
```

**FLASH:**

```
>time flash -t 1 -O -M 1000 -x 0.1 -o merge.assembled illumina_ecoli_ART1.fq
illumina_ecoli_ART2.fq
[FLASH] Starting FLASH v1.2.11
[FLASH] Fast Length Adjustment of SHort reads
[FLASH]
[FLASH] Input files:
[FLASH]     ../../illumina_ecoli_ART1.fq
[FLASH]     ../../illumina_ecoli_ART2-2.fq
[FLASH]
[FLASH] Output files:
[FLASH]     ./merge.assembled.extendedFrags.fastq
[FLASH]     ./merge.assembled.notCombined_1.fastq
[FLASH]     ./merge.assembled.notCombined_2.fastq
[FLASH]     ./merge.assembled.hist
[FLASH]     ./merge.assembled.histogram
[FLASH]
[FLASH] Parameters:
[FLASH]     Min overlap:          10
[FLASH]     Max overlap:          1000
[FLASH]     Max mismatch density:  0.100000
[FLASH]     Allow "outie" pairs:   true
[FLASH]     Cap mismatch quals:    false
[FLASH]     Combiner threads:      1
[FLASH]     Input format:         FASTQ, phred_offset=33
[FLASH]     Output format:        FASTQ, phred_offset=33
[FLASH]
[FLASH] Starting reader and writer threads
[FLASH] Starting 1 combiner threads
[FLASH] Processed 25000 read pairs
[FLASH] Processed 50000 read pairs
[FLASH] Processed 75000 read pairs
[FLASH] Processed 100000 read pairs
[FLASH] Processed 125000 read pairs
```

```
[FLASH] Processed 150000 read pairs
[FLASH] Processed 175000 read pairs
[FLASH] Processed 200000 read pairs
[FLASH] Processed 225000 read pairs
[FLASH] Processed 250000 read pairs
[FLASH] Processed 275000 read pairs
[FLASH] Processed 300000 read pairs
[FLASH] Processed 325000 read pairs
[FLASH] Processed 350000 read pairs
[FLASH] Processed 375000 read pairs
[FLASH] Processed 400000 read pairs
[FLASH] Processed 425000 read pairs
[FLASH] Processed 450000 read pairs
[FLASH] Processed 475000 read pairs
[FLASH] Processed 500000 read pairs
[FLASH] Processed 525000 read pairs
[FLASH] Processed 550000 read pairs
[FLASH] Processed 575000 read pairs
[FLASH] Processed 600000 read pairs
[FLASH] Processed 625000 read pairs
[FLASH] Processed 650000 read pairs
[FLASH] Processed 675000 read pairs
[FLASH] Processed 700000 read pairs
[FLASH] Processed 725000 read pairs
[FLASH] Processed 750000 read pairs
[FLASH] Processed 775000 read pairs
[FLASH] Processed 800000 read pairs
[FLASH] Processed 825000 read pairs
[FLASH] Processed 850000 read pairs
[FLASH] Processed 875000 read pairs
[FLASH] Processed 900000 read pairs
[FLASH] Processed 925000 read pairs
[FLASH] Processed 950000 read pairs
[FLASH] Processed 975000 read pairs
[FLASH] Processed 1000000 read pairs
[FLASH]
[FLASH] Read combination statistics:
[FLASH]     Total pairs:      1000000
[FLASH]     Combined pairs:   326686
[FLASH]         Innie pairs:   326626 (99.98% of combined)
[FLASH]         Outie pairs:   60 (0.02% of combined)
[FLASH]     Uncombined pairs: 673314
[FLASH]     Percent combined: 32.67%
[FLASH]
```

```
[FLASH] Writing histogram files.
[FLASH]
[FLASH] FLASH v1.2.11 complete!
[FLASH] 52.891 seconds elapsed

real    0m52.914s
user    0m53.356s
sys     0m3.324s
```

**PEAR**

```
>time pear -f illumina_ecoli_ART1.fq -r illumina_ecoli_ART2.fq -o pear.merged
 ____  _____  _        ____
|  _ \| ____|  / \  |  _ \
| |_) |  _|   / _ \ | |_) |
|  __/| |___ / ___ \|  _ <
|_|   |_____/_/   \_\_| \_\

PEAR v0.9.8 [April 9, 2015]

Citation - PEAR: a fast and accurate Illumina Paired-End reAd mergeR
Zhang et al (2014) Bioinformatics 30(5): 614-620 | doi:10.1093/bioinformatics/btt593

Forward reads file.................: illumina_ecoli_ART1.fq
Reverse reads file.................: illumina_ecoli_ART2.fq
PHRED..............................: 33
Using empirical frequencies........: YES
Statistical method.................: OES
Maximum assembly length............: 999999
Minimum assembly length............: 50
p-value............................: 0.010000
Quality score threshold (trimming).: 0
Minimum read size after trimming...: 1
Maximal ratio of uncalled bases....: 1.000000
Minimum overlap....................: 10
Scoring method.....................: Scaled score
Threads............................: 1

Allocating memory..................: 200,000,000 bytes
Computing empirical frequencies....: DONE
  A: 0.246168
  C: 0.253836
  G: 0.253816
  T: 0.246180
```

```
  0 uncalled bases
Assemblying reads: 100%

Assembled reads ...................: 995,648 / 1,000,000 (99.565%)
Discarded reads ...................: 0 / 1,000,000 (0.000%)
Not assembled reads ...............: 4,352 / 1,000,000 (0.435%)
Assembled reads file...............: pear.merged.assembled.fastq
Discarded reads file...............: pear.merged.discarded.fastq
Unassembled forward reads file.....: pear.merged.unassembled.forward.fastq
Unassembled reverse reads file.....: pear.merged.unassembled.reverse.fastq

real    22m42.668s
user    22m41.456s
sys    0m1.184s
```

**COPE**

```
>time cope -a illumina_ecoli_ART1.fq -b illumina_ecoli_ART2.fq -o cope_full_mode.fq -2
left1.fq -3 left2.fq -s 33 -u 1000 -c 0.9 -m 3 -t kmer-cope.freq.cz -f kmer-cope.freq.cz.len
Program start..
Program:        COPE (Connecting Overlapped Pair-End reads)
Version:        v1.1.2
Author:                 BGI-ShenZhen
CompileDate:  Jul 20 2016 time: 14:35:36
Current time:   Mon May 14 20:18:48 2018
Command line:           cope -a ../../illumina_ecoli_ART1.fixed.fq
-b ../../illumina_ecoli_ART2-2.fq -o cope_full_mode.fq -2 left1.fq -3 left2.fq -s 33 -u 1000 -c
0.9 -m 3 -t ../../kmer-cope.freq.cz -f ../../kmer-cope.freq.cz.len
loading kmerfreq table...
kmer freq table:
kmer_num        node_num        error_freq_num          error_ratio       suspicious_freq_num
          suspicious_ratio          normal_freq_num        normal_ratio
          repeat_freq_numrepeat_ratio
468304845       94053426        88334798        0.939198        1250904          0.0132999
          178635            0.00189929     4289089          0.0456027
finish loading kmer freq table!
 Run time: 11s.
Begin read files and connect pairs...
Process pair reads number: 1000000
Connect reads finished!
Kmer frequency based connection table:
```

```
total_pairs	connected_pairs	connect_ratio(%)	low_quality_pairs	low_quality_ratio(%)
1000000	292303	29.2303	0	0
starting to do cross connect for 707697 pair reads
Use connected file to cross
Count pair kmer number...
Count kmer pair number: 0
loading pair kmer information!
starting to load pair kmer file...
loading kmer file finished! Totally load kmer pair number is: 0
sort pair kmers!
load hashset!
final hash set size is 1048583
load all reads to get cross reads
Begin load read file: cope_full_mode.fq
finished load read file: cope_full_mode.fq ! totally load 292303 reads
Get cross read finished and totally cross 0 reads!
Begin to cross connect reads, processing file: cross.read.inf0.gz ...
No cross reads, cross reads connect finished!
All done!

Cross connection table:
total_pairs	no_marker	have_cross	have_cross_ratio(%)	have_cross_connect	cross_connect_ratio(%)
0	0	0	-nan	0	-nan
All done, Thank you!
 Run time: 469s.

real	7m48.480s
user	7m28.328s
sys	0m19.956s
```

**BBMerge:**

```
time bbmerge.sh in1=illumina_ecoli_ART1.fixed.fq in2=illumina_ecoli_ART2-2.fq
out=bbmerge_assembled.fastq outu1=bbmerge_unassembled1.fastq
outu2=bbmerge_unassembled2.fastq minoverlap=10 maxratio=0.1

Version 38.01 [in1=illumina_ecoli_ART1.fixed.fq, in2=illumina_ecoli_ART2-2.fq,
out=bbmerge_assembled.fastq, outu1=bbmerge_unassembled1.fastq,
outu2=bbmerge_unassembled2.fastq, minoverlap=10, maxratio=0.1]

Writing mergable reads merged.
Started output threads.
```

Total time: 24.879 seconds.

| | | |
|---|---|---|
| Pairs: | 1000000 | |
| Joined: | 201842 | 20,184% |
| Ambiguous: | 768588 | 76,859% |
| No Solution: | 29570 | 2,957% |
| Too Short: | 0 | 0,000% |

| | |
|---|---|
| Avg Insert: | 401.8 |
| Standard Deviation: | 10.1 |
| Mode: | 401 |

| | |
|---|---|
| Insert range: | 104 - 491 |
| 90th percentile: | 415 |
| 75th percentile: | 409 |
| 50th percentile: | 402 |
| 25th percentile: | 395 |
| 10th percentile: | 389 |

| | |
|---|---|
| real | 0m26.408s |
| user | 1m43.780s |
| sys | 0m1.900s |

## Results validation

Blast was used in order to validate the results generated by each of the merger tools
First, we created a database of the reference genome fasta file:

```
>formatdb -p F -i ecoli_K-12.fasta
```

Second, we compared all the merged sequences against the created database.

```
>blastall -p blastn -d ecoli_K-12.fasta -i ../overlapped.fastq -a 2 -m 9 -o blast_output
```

An in-house script was used to summarize all the best hits from each merged sequence.

The results of OverlapPER are also available on
https://sourceforge.net/projects/overlapper-reads/

## PIPEBAR results

PIPEBAR has been tested over the dataset presented above. The comparison of PIPEBAR results and the others softwares are presented in Table 1 of the main manuscript. We submitted fastq files to FastQC, in order to verify the quality of the generated sequences. Given that only PIPEBAR and Geneious generate FASTQ files as output, we only compare their results.

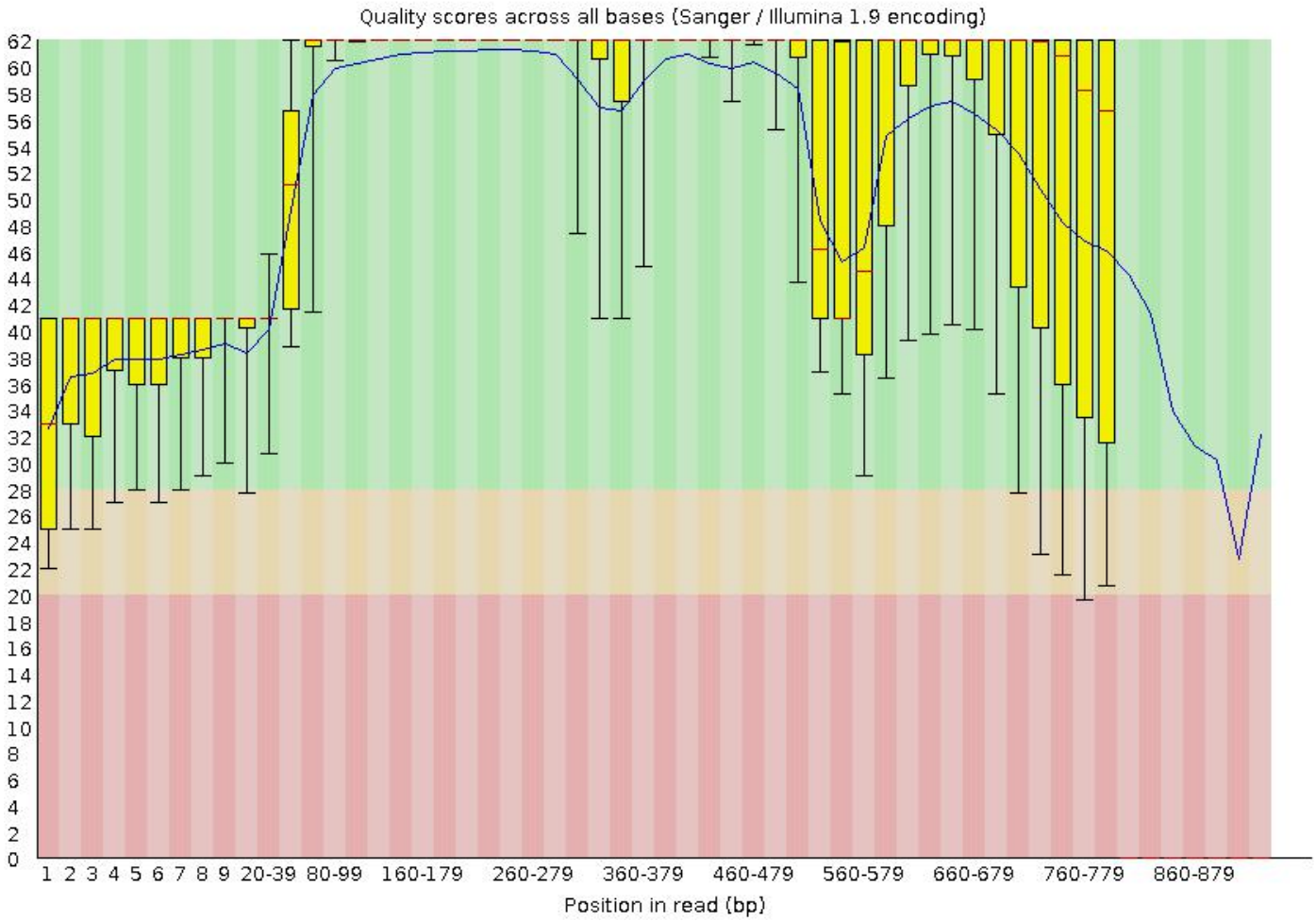



Figure S1 - Box diagram of bases quality for all sequences generated by PIPEBAR.

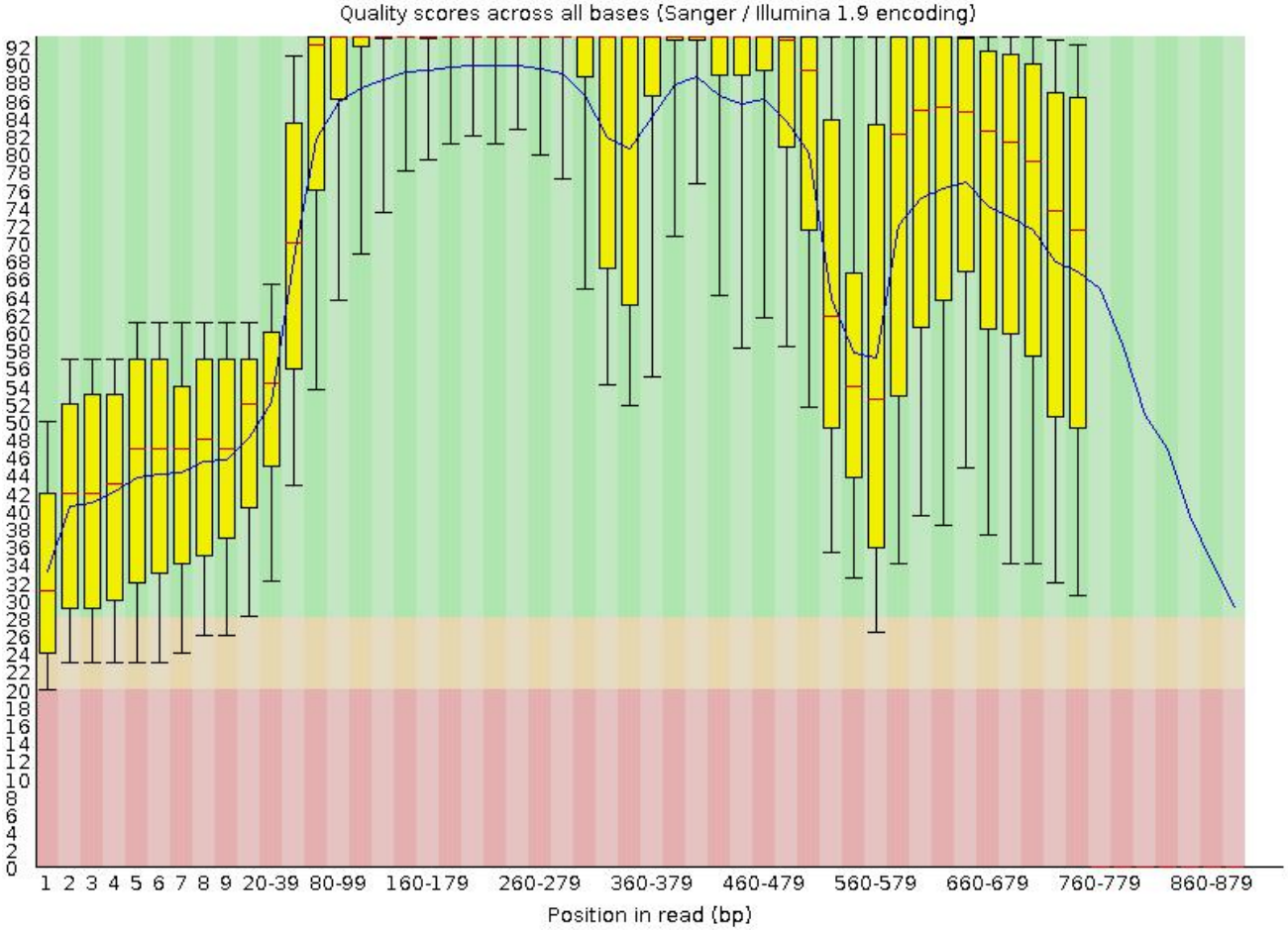



Figure S2 - Box diagram of bases quality for all sequences generated by Geneious.

Figures S1 and S2 show that the overall quality bases from the sequences generated by PIPEBAR are very similar to the quality generated by our benchmark (Geneious).

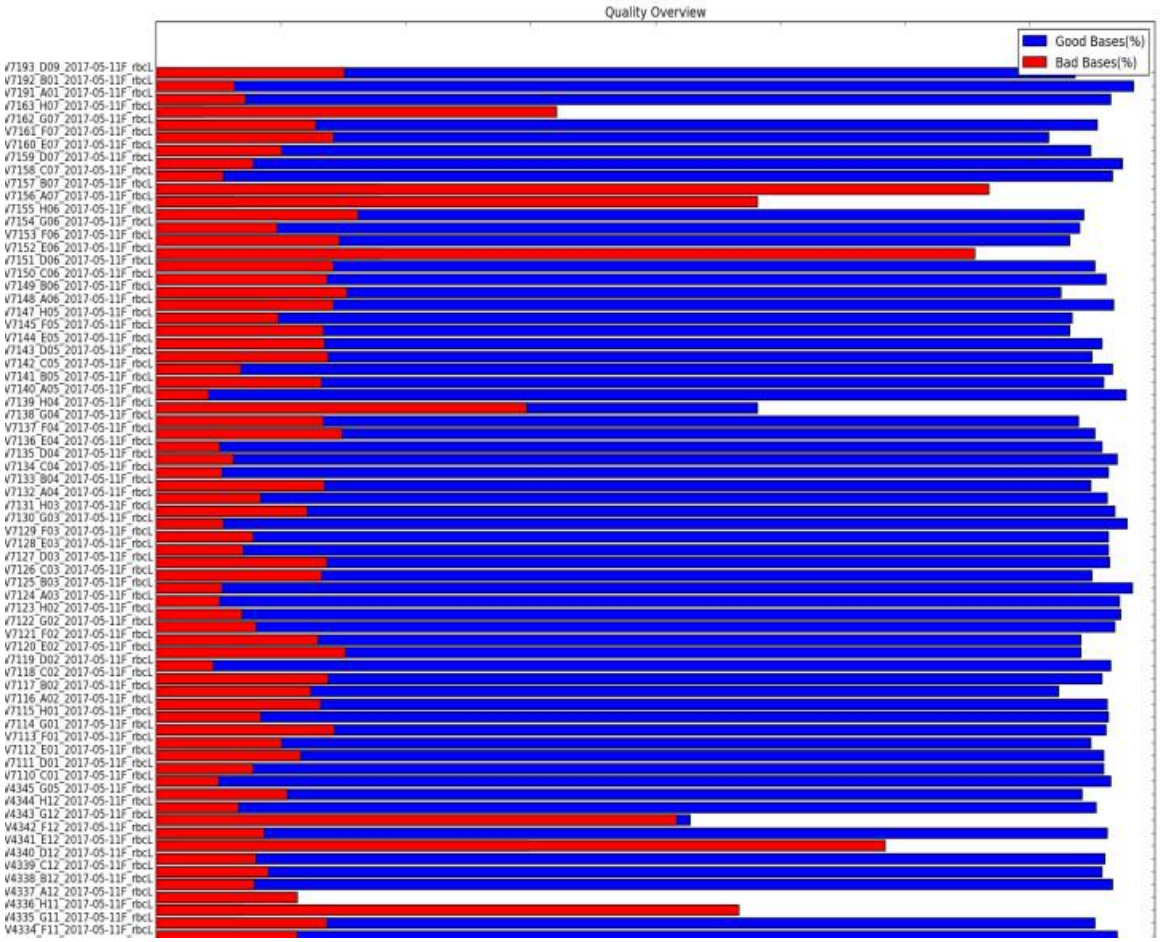



Figure S3 - Quality report generated by Pipebar. Each bar represents the quality of the bases from a barcode. The red color indicates quality below the specified PHRED threshold, while blue color indicates quality above that threshold.