

NxRepair: Error Correction in De Novo Sequence Assembly Using Nextera Mate Pairs

Rebecca R. Murphy¹, Jared O’Connell², Anthony J. Cox², and Ole Schulz-Trieglaff²

¹Department of Chemistry, University of Cambridge, UK

²Illumina Cambridge, Chesterford Research Park, Essex, CB10 1XL

ABSTRACT

Scaffolding errors and incorrect repeat disambiguation during *de novo* assembly can result in large scale misassemblies in draft genomes. Nextera mate pair sequencing data provide additional information to resolve assembly ambiguities during scaffolding. Here, we introduce NxRepair, an open source toolkit for error correction in *de novo* assemblies that uses Nextera mate pair libraries to identify and correct large-scale errors. We show that NxRepair can identify and correct large scaffolding errors, without use of a reference sequence, resulting in quantitative improvements in the assembly quality. NxRepair can be downloaded from GitHub; a tutorial and user documentation are also available.

Keywords: de novo assembly, mate pair, genome assembly, error correction, scaffolding, insert size, misassembly, misassembly correction, assembly quality, automated error detection

INTRODUCTION

De novo assembly is the construction of a long, contiguous genomic sequence from short DNA reads, without using a reference genome. A common method of *de novo* genome assembly is construction and traversal of a De Bruijn graph (Compeau et al., 2011) formed by combining overlapping short reads. In the simplest case, the graph is constructed from single end reads. However, with only single end reads, disambiguating repeat regions, which tangle the de Bruijn graph, remains challenging. Paired end read technology (Fullwood et al., 2009), in which a longer fragment of DNA is sequenced from both ends, to create a pair of short reads separated by an unsequenced region. The genomic distance from the start of one read to the end of the other is termed the insert size. Paired end reads with insert sizes of a few hundred bases provide some additional information for repeat disambiguation; even more useful are read pairs with a large insert size of several kilobases, such as the Illumina Nextera mate pairs (Illumina, 2012). Assembly is typically a two-stage process. First, long contiguous sections, named contigs, are constructed. Second, once the contigs cannot be extended any further, scaffolding algorithms attempt to join multiple contigs, using insert size information to determine contig order and approximate gap size. Many assemblers incorporate mate pair insert size information into either both the contig assembly and scaffolding processes (Bankevich et al., 2012), or just into the scaffolding step (Zerbino and Birney, 2008) but errors can still occur. The most serious mistakes are large scale scaffolding or extension errors (Fig. 1 (A)), in which two disparate regions of a genome are incorrectly joined together. Similarly, large insertion or deletion errors (indels) create structural irregularities in the *de novo* assembly; whereas mistakes in base calling lead to errors at a single position only.

Errors in *de novo* assembly can significantly affect the quality of the assembled genome, with repercussions for downstream research. Consequently, error correction and quality evaluation of *de novo* assemblies are problems receiving considerable research interest. Recent work, such as the Assemblathon (Bradnam et al., 2013) and GAGE (Salzberg et al., 2012) collaborations, compare the quality of assemblies prepared

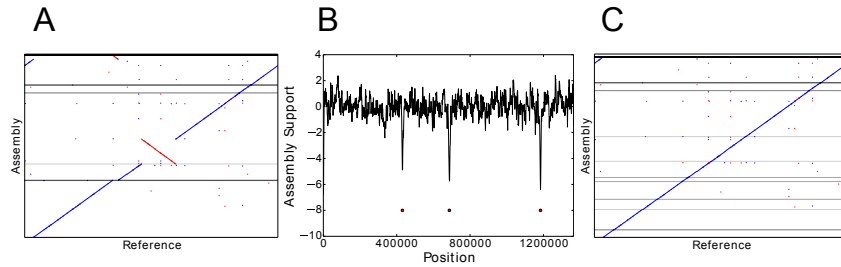


Figure 1. Using NxRepair to remove large misassemblies. (A) Alignment of the *de novo* assembly of the TB genome to its reference genome. The assembly contains several large misassemblies. (B) A plot of NxRepair’s support metric against scaffold position for the TB assembly. Low support for the assembly is identified in three regions of a contig. (C) Breaking the contigs at the identified positions resolves the most significant misassemblies. In (A) and (C), horizontal lines demarcate the scaffold boundaries.

by various assemblers by comparing the *de novo* assemblies produced with their equivalent reference genomes. However, in the absence of a high-quality reference genome, other methods of quality evaluation must be used. Ghodsi et al. (Ghodsi et al., 2013) have developed a Bayesian method of assembly quality evaluation, which can calculate an assembly quality score, without requiring a reference genome. However, this provides only an overall quality score and cannot be used to identify errors or low-quality regions. Several recent papers have developed error identification and correction methods, which perform a fine-grained analysis of assembly quality. The most well-known of these is the A5 Assembly Pipeline (Coil et al., 2014; Tritt et al., 2012), which includes an error detection and resc scaffolding step that makes use of mate pair alignment information. Two new tools, REAPR (Hunt et al., 2013) and ALE (Clark et al., 2013) have also been developed to use read pair data to identify misassemblies. A similar tool is currently under development at the Broad Institute (Walker, 2014). However, with the exception of ALE, which is no longer actively maintained, these newer tools are not optimised to use mate pair information.

Here we introduce NxRepair, an assembly error detection tool that can identify the most serious misassemblies by examining the distribution of Nextera mate pair insert sizes. NxRepair does not require a reference genome and can be used with assemblies prepared with just a single mate pair library. It specifically targets the most serious scaffolding errors and large-scale indels by identifying regions with a high number of anomalous insert sizes, or very few supporting reads, breaking the scaffold and optionally trimming out the misassembled region. NxRepair also provides a fine-grained quality score, allowing researchers to visualise poor-quality regions. We demonstrate usage of NxRepair on bacterial genomes assembled from a single Nextera mate pair library using the state of the art SPAdes assembler (Bankevich et al., 2012), which explicitly uses insert size information during contig construction, as well as for scaffolding. Using these genomes, we benchmark NxRepair against the error correction module of the A5 assembler, A5qc (Tritt et al., 2012), which is currently the most widely used error correction tool, demonstrating NxRepair’s superior performance.

IMPLEMENTATION

Statistical Analysis of Mate Pair Insert Sizes

Nextera mate pair libraries are prepared to have a certain insert size, typically between 1 and 10 kb. When the mate pairs used to prepare an assembly are aligned back to the assembly, large misassemblies result in unusual insert sizes and read orientations. We model this using a two-component mixture distribution. The first component of this mixture is the insert size distribution of correctly aligned mate pairs. We model the distribution of insert sizes, Y , as a normal distribution with mean $\hat{\mu}$ and standard deviation $\hat{\sigma}$: $Y \sim N(\hat{\mu}, \hat{\sigma}^2)$. We estimate $\hat{\mu}$ and $\hat{\sigma}$ for the entire genome by aligning reads back to the assembly and using robust estimators (see below). The second component, defined as a uniform distribution across the contig size $U(0, L)$ for a contig of length L , captures anomalous insert sizes.

A uniform distribution was selected to model insert sizes, as it makes no assumption about the cause of an

anomaly.

To calculate the degree of support for the assembly at each site across a contig, NxRepair retrieves all mate pairs spanning the region $[i - W, i + W]$, of size $2W - 1$ at position i on the contig, where spanning is defined to mean that one read ends entirely before the region $[i - W, i + W]$ and the other read begins entirely after (see Fig. 2.) The default value of W is 200 bases (see Table 2). We define a latent indicator variable $X_l \in \{0, 1\}$ for each pair of reads, l , which takes the value 1 if the insert size came from the null distribution, and 0 otherwise. Within each window queried, the probability that each retrieved read, r_l is drawn from the null distribution is given by:

$$P(X_l = x | Y_l) = \frac{P(X_l = x)(Y_l | X_l = x)}{\sum_{k=0}^1 P(X_l = k)(Y_l | X_l = k)} \quad (1)$$

$$= \frac{\pi_x(Y_l | X_l = x)}{\sum_{k=0}^1 \pi_k(Y_l | X_l = k)} \quad (2)$$

where Y_l is the insert size of read pair l , π_x is the user defined prior probability of class x and $\pi_1 + \pi_0 = 1$. The default value of π_0 is 0.01 (see Table 2), meaning that in the absence of any insert size information, 99 % of read pairs are expected to arise from the null distribution.

Within each window, the total support for a correct assembly at position i can be calculated as:

$$D_i = \sum_{l=1}^N P(X_l = 1 | Y_l) \cdot C_l \quad (3)$$

where the summation is over all read pairs aligning across position i and C_l is an indicator variable, reporting pairing orientation:

$$C_l = \begin{cases} 1, & \text{if mate pairs have correct orientation and strand alignment} \\ 0, & \text{otherwise} \end{cases} \quad (4)$$

Within each contig, the contig assembly support mean μ_D and variance s_D are calculated from all reads aligning to the contig,

$$\hat{\mu}_D = \frac{\sum_{l=1}^N D_l}{N} \quad s_D = \frac{\sum_{l=1}^N \sqrt{(D_l - \hat{\mu})^2}}{N} \quad (5)$$

We use these contig specific mean and variance, rather than the global values, to prevent local variations in coverage from either causing false positives or masking changes in the insert size distribution. Using these values, the Z-score z_l within each queried interval is calculated as:

$$z_l = \frac{D_l - \hat{\mu}_D}{s_D} \quad (6)$$

The Z-score is sensitive both to local changes in the insert size distribution, and to large variations in the number of correctly aligned mate pairs, for example caused by a large number of reads with a mate aligning to a different contig. This ensures that NxRepair can identify misassemblies occurring both within and between contigs.

A misassembly is identified if $z_l < T$ for a user-defined threshold T (default value -4). This threshold describes the number of standard deviations below the mean assembly support that is required to identify an anomaly. The default value of -4 will flag only positions whose assembly support is less than four standard deviations below the mean level of support.

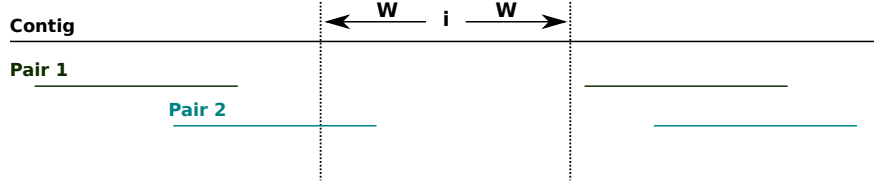


Figure 2. Schematic illustrating mate pairs spanning a selected region. The upper read pair (green) spans the indicated region, as both reads align entirely outside of the region $[i - W, i + W]$. The lower read pair (cyan) do not span the indicated region, as the left-hand read overlaps the target area.

Global Assembly Parameters

NxRepair identifies misassemblies by identifying regions where the mate pair insert size distribution differs significantly from the insert size distribution across the entirety of the *de novo* assembly. Consequently, it is necessary to have a robust estimate of the global mate pair insert size distribution. For calculation of population statistics, mate pairs that align to different contigs are excluded, as are mate pairs with an incorrect strand or pairing orientation, pairs whose mapping quality falls below a user specified threshold, and pairs whose insert size exceed 30 kb (approximately 10 times the mean insert size). The global mean $\hat{\mu}$ and median absolute deviation MAD are calculated across all contigs in the assembly as:

$$\hat{\mu} = \frac{\sum_{l=1}^N Y_l}{N} \quad \text{MAD} = \text{median}(|Y_l - \text{median}(Y_l)|) \quad (7)$$

where Y_l is the insert size of the l th of N reads with correct pairing behaviour. The standard deviation was then calculated from the MAD, using:

$$\hat{\sigma} = K \cdot \text{MAD} \quad (8)$$

for $K = 1.4826$

The MAD is a robust estimator for the standard deviation, as it is not sensitive to outliers, such as the long tail of the mate pair insert size distribution. Using the MAD as an estimator prevents over-estimating the variance of the insert size, allowing anomalously large insert sizes to be correctly identified.

These were then used as the parameters of the null distribution, as described in the main paper.

Interval Tree Construction

To facilitate rapid lookup of mate pair properties, we construct an interval tree (Cormen et al., 2009) for each contig in the *de novo* assembly. An interval tree is a data structure that facilitates $O(\log n + m)$ lookup of intervals that span a given point or interval, for n total entries and m spanning entries. The interval tree contains the start and end positions of each mate pair aligned to that contig, as well as a flag variable indicating whether that mate pair had correct strand and pairing orientation. Mate pairs where the two reads align to different contigs were excluded. For each position i for which the Z-score is to be calculated, the tree is queried with a start position $i - W$ and end position $i + W$, to retrieve read pairs spanning the interval between positions $i - W$ and $i + W$ (exclusive). The insert sizes of retrieved read pairs are then used to calculate the Z-score for position i . This allows NxRepair to rapidly query positions across a contig to discover the insert size distribution at the queried position. Use of the interval tree significantly increases the efficiency of Z-score calculation, as each pair of reads is fetched only once from the bam file in order to build the tree. All relevant parameters are then stored in the tree for rapid look-up when a position is queried. This has several advantages. Firstly, it is significantly faster than fetching reads only when a position is queried. Secondly, it is more space efficient than a frequency array of all positions on all contigs but does not lose any information about the exact alignment positions. Finally,

once construction of the tree is complete, multiple passes across a contig (for example with different spatial resolution, or using different window sizes) can rapidly be made using the same tree.

Misassembly Location and Contig Breaking

To improve the quality of the *de novo* assembly, a contig is broken into two separate pieces at the site of a misassembly. The broken ends of the two new contigs can optionally be trimmed by a user defined length (default 4 kb) to remove the misassembled region. Trimming allows removal of the incorrectly assembled regions around a break-point, but can be switched off if a user does not want any sequence to be removed from the assembly. To prevent excessive clipping, misassemblies separated by less than the trimming distance are grouped together, the contig is broken at the start and end of the misassembled region and the misassembled section is discarded. Low-scoring regions within the trimming distance of the ends of contigs are not considered misassemblies, as the high proportion of mate pairs aligning here whose mate maps to a different contig reduces the number of pairs under consideration and hence lowers the observed Z-score. This also ensures that circular molecules, such as small plasmids, which are assembled into a single contig, are not truncated because of mate pairs at either end of the assembly that appear to span the entire contig, but which are spatially close when circularisation is considered.

Availability and Dependencies

NxRepair is available for free anonymous download from the Python Package Index (PyPI) here: <https://pypi.python.org/pypi/nxrepair>. The source code, written in python is hosted on GitHub: <https://github.com/rebeccaroisin/nxrepair>. A full tutorial and API can be found on ReadTheDocs: <http://nxrepair.readthedocs.org/en/latest/>.

NxRepair makes use of several further open source libraries, specifically:

Numpy (van der Walt et al., 2011) (<http://www.numpy.org/>)

Scipy (Millman and Aivazis, 2011) (<http://www.scipy.org/>)

Matplotlib (Hunter, 2007) (<http://matplotlib.org/>)

Pysam (<https://pypi.python.org/pypi/pysam>), the python wrapper for Samtools

Samtools (Li et al., 2009) (<http://samtools.sourceforge.net/>)

We installed the numpy, scipy and matplotlib libraries via Anaconda (<https://store.continuum.io/cshop/anaconda/>).

We have used the Interval Tree implementation from the bx-python library (https://bitbucket.org/james_taylor/bx-python/wiki/Home).

MATERIALS AND METHODS

Data

Nine bacterial genomes were prepared according to the Nextera mate pair protocol and sequenced in a single MiSeq run using 2×151 bp reads. The genomes sequenced are shown in Table 4. Reads were trimmed using the MiSeq inbuilt trimmer. The untrimmed reads are available from BaseSpace via <https://basespace.illumina.com/s/TXv32Ve6wTl9> (free registration required). Note that only these Nextera mate pair libraries were used. No additional single end or paired end libraries were required.

Performance Optimisation

ROC Plots

To optimise the threshold in Z below which to identify a misassembled region, we prepared ROC plots, varying the threshold value, T , in steps of 1 between -10 and 0.

Genome	Before NxRepair		After NxRepair		After A5qc	
	No.	NGA50	No.	NGA50	No.	NGA50
Bcer	0	1157846	0	1157846	0	1157846
EcDH	7	573003	6	573003	7	573003
EcMG	3	693692	3	693692	3	693692
List	0	1496613	0	1496613	0	1496613
Meio	0	2702549	0	2702549	0	2702549
ped	1	1269147	0	952558	1	1269147
pneu	8	578813	8	578813	15	647909
Rhod	8	2715434	8	2715434	8	2715434
TB	63	186136	57	186136	63	186136

Table 1. Number of large misassemblies and NGA50 as reported by QUAST before and after NxRepair correction.

The positions of true misassemblies were identified by aligning each *de novo* assembly to its reference genome using QUAST (Gurevich et al., 2013). To correctly compare the sites of true misassemblies with those identified by NxRepair, we divided each contig of the assembly into short stretches of 1 kb length. We then prepared an array, A_{Nx} of size $\frac{L}{1000}$ for contig length L , corresponding to misassemblies identified by NxRepair. A_{Nx} was filled as follows:

$$A_{Nx} = \begin{cases} 1, & \text{if NxRepair identified a misassembly in stretch } i \\ 0, & \text{otherwise} \end{cases} \quad (9)$$

To prepare the ROCs each position i in A_{Nx} was labeled as true positive (TP) if $A_{Nx}[i] = 1$ and a true misassembly fell within it, true negative (TN) if $A_{Nx}[i] = 0$ and no true misassembly occurred within the interval, false positive (FP) if $A_{Nx}[i] = 1$ but no true misassembly had occurred, or false negative (FN) if $A_{Nx}[i] = 0$ but the interval contained a true misassembly. The true positive rate (TPR) and false positive rate (FPR) were then calculated as follows:

$$\text{TPR} = \frac{\text{TP}}{\text{TP} + \text{FN}} \quad \text{FPR} = \frac{\text{FP}}{\text{FP} + \text{TN}} \quad (10)$$

Based on the resultant ROC plots, shown in Fig. 3, a threshold in Z of -4 was found to detect true misassemblies with minimal false positives, so was used for all subsequent analyses.

Profiling

Performance analysis was performed on a single core with 8 GB RAM available. Runtime analysis was performed using the python cProfile module. The memoryprofiler python module was used to analyse memory usage.

Workflow Pipeline

De novo assemblies were prepared using the SPAdes Assembler, version 3.1.1 (Bankevich et al., 2012):

```
spades.py -k 21,33,55,77 -t 4
--hqmp1-12 bacteria.fastq.gz --hqmp1-fr -o assembly
```

The initial assembly quality was evaluated using QUAST (Gurevich et al., 2013) (version 2.3) to align the *de novo* assembly to a reference genome:

```
python quast.py -o results.sample -t 16
-R ref/reference.fna sample.new.fasta
```

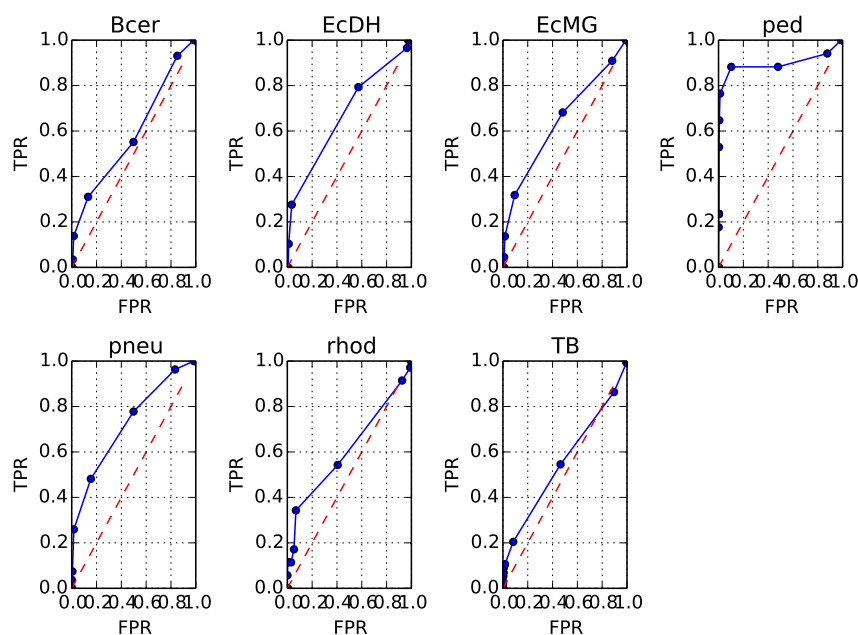


Figure 3. ROC plots for the seven genomes containing misassemblies.

Following assembly, the same reads used to generate the assembly were aligned back to the *de novo* assembly using BWA-MEM (Li, 2013) (BWA version 0.7.10). A sorted BAM file of the resulting alignment was then prepared using SAMtools (version 1.1) (Li et al., 2009):

```
bwa index sample/scaffolds.fasta
bwa mem sample/scaffolds.fasta -p bacteria.fastq.gz | samtools view -bS
- | samtools sort - sample
samtools index sample.bam
```

We identified misassemblies using NxRepair (version 0.13) as follows:

```
python nxrepair.py sample.bam sample/scaffolds.fasta sample_scores.csv
sample_new.fasta -img_name sample_new
```

The default parameters used and their meanings are shown in Table 2. These have been optimised for Illumina Nextera mate pair libraries with a mean insert size of approximately 4 kb. For mate pair libraries with a much larger (smaller) insert size, the maxinsert and trim parameters may need to be increased (decreased).

Finally we used QUAST (Gurevich et al., 2013) to evaluate the assembly quality following NxRepair by aligning the *de novo* assembly to a reference genome as described above.

RESULTS AND DISCUSSION

We used NxRepair to correct *de novo* assemblies from nine bacterial genomes. The genomes used are described above. Mate pair reads were trimmed, assembled using the SPAdes assembler (version 3.1.1) (Bankevich et al., 2012) and then aligned back to the assembled scaffold using BWA-MEM (Li, 2013). We used QUAST (Gurevich et al., 2013) to evaluate the assembly quality before and after NxRepair correction by aligning to an appropriate reference genome. For all NxRepair analyses, the default parameters, shown in Table 2 were used. Fig. 1 (A) shows a misassembled genome that contained several scaffolding errors identified by NxRepair (Fig. 1 (B)). Following NxRepair correction, the most significant structural misassemblies were resolved (Fig. 1 (C)). The improvement following NxRepair

Parameter	Default Value	Meaning
imgname	None	Prefix under which to save plots.
maxinsert	30000	Maximum insert size, below which a read pair is included in calculating population statistics.
minmapq	40	Minimum MapQ value, above which a read pair is included in calculating population statistics.
minsize	10000	Minimum contig size to analyse.
prior	0.01	Prior probability that the insert size is anomalous.
stepsize	1000	Step-size in bases to traverse contigs.
trim	4000	Number of bases to trim from each side of an identified misassembly.
T	-4.0	Threshold in Z score (number of standard deviations from the mean) below which a misassembly is called.
window	200	Window size across which bridging mate pairs are evaluated.

Table 2. NxRepair Parameters

Bacterium	Total Time (s)	Memory Usage (MiB)
Bcer	78	271
EcDH	123	444
EcMG	70	260
list	97	383
meio	259	565
ped	123	417
pneu	59	227
rhod	190	463
TB	155	411

Table 3. NxRepair performance analysis.

correction is shown for all nine genomes in Table 1. For two assemblies, errors were removed without reducing NGA50; for one genome, errors were removed but NGA50 was slightly reduced; for six genomes, three of which contained no large errors, no errors were found and the assembly was unchanged. We are not able to correct all misassemblies, as not all misassemblies exhibit a change in Z-score large enough to identify an error against the background score fluctuation caused by the wide insert size distribution of the Nextera mate pairs.

Performance

We evaluated the runtime and peak memory usage of NxRepair on each of the nine genomes analysed. The results are shown in Table 3. The most memory and computationally intensive part of the NxRepair analysis is construction of the interval trees. The size of each interval tree is dependent on the contig size. Consequently, we expect both runtime and memory usage to scale with the size of the largest contig.

CONCLUSIONS

NxRepair is a simple error correction module that can be used to identify and remove large scale errors from *de novo* assemblies using Nextera mate pair reads. We evaluated NxRepair using *de novo* assemblies nine bacterial genomes prepared using the SPAdes assembler, showing that of the six genomes containing misassemblies, three could be improved by NxRepair correction; compared with no improvements made by the A5qc module. SPAdes is the current state of the art in bacterial genome assembly and explicitly uses mate pair information during both contig construction and scaffolding. Even in these excellent assemblies, NxRepair could identify misassemblies and improve the assembly quality. We predict that NxRepair will

Abbreviation:	Bcer
Bacteria:	<i>Bacillus cereus</i> ATCC 10987
Accession ID:	NC.003909, NC.005707
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Bacillus_cereus_ATCC_10987_uid57673/
Abbreviation:	EcDH
Bacteria:	<i>Escherichia coli</i> str. K-12 substr. DH10B
Accession ID:	NC.010473
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Escherichia_coli_K_12_substr__DH10B_uid58979/
Abbreviation:	EcMG
Bacteria:	<i>Escherichia coli</i> str. K-12 substr. MG1655
Accession ID:	NC.000913
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Escherichia_coli_K_12_substr__MG1655_uid57779/
Abbreviation:	list
Bacteria:	<i>Listeria monocytogenes</i>
Accession ID:	NC.003210
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Listeria_monocytogenes_EGD_e_uid61583/
Abbreviation:	meio
Bacteria:	<i>Meiothermus ruber</i> DSM 1279
Accession ID:	NC.013946
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Meiothermus_ruber_DSM_1279_uid46661/
Abbreviation:	ped
Bacteria:	<i>Pedobacter heparinus</i> DSM 2366
Accession ID:	NC.013061
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Pedobacter_heparinus_DSM_2366_uid59111/
Abbreviation:	pneu
Bacteria:	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> MGH 78578
Accession ID:	NC.009648, NC.009649, NC.009650, NC.009651, NC.009652, NC.009653
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Klebsiella_pneumoniae_MGH_78578_uid57619/
Abbreviation:	rhod
Bacteria:	<i>Rhodobacter sphaeroides</i> 2.4.1
Accession ID:	NC.007488, NC.007489, NC.007490, NC.007493, NC.007494, NC.009007, NC.009008
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Rhodobacter_sphaeroides_2_4_1_uid57653/
Abbreviation:	TB
Bacteria:	<i>Mycobacterium tuberculosis</i> H37Ra
Accession ID:	NC.009525
NCBI FTP:	ftp.ncbi.nih.gov/genomes/Bacteria/Mycobacterium_tuberculosis_H37Ra_uid58853/

Table 4. Summary of bacteria analysed and the relevant NCBI information on their reference genomes. There were two repeats of each strain. All 18 samples were prepared with the Nextera mate pair protocol and sequenced in a single MiSeq run using 2×151 bp reads. The untrimmed reads we used as input to NxTrim (3.9Gbp in all) are available from BaseSpace via <https://basespace.illumina.com/s/TXv32Ve6wTl9> (free registration required).

be even more useful for identifying errors in *de novo* assemblies where mate pair information was used only at the scaffolding stage. NxRepair is freely available online and can be run with a single call from the command line, making it an attractive option for improving assembly quality.

ACKNOWLEDGMENTS

We thank Emma Carlson and Niall Gormley (Illumina Cambridge) for preparing the Nextera mate pair libraries.

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