1 Introduction

With whole genome sequencing it is still rather expensive to achieve sufficient read coverage for example for the detection of genomic variants. Further, in some cases one might be interested only in some fraction rather than the whole genome, for example linkage regions or the complete exome. Target capture (target enrichment, targeted sequencing) experiments are a suitable strategy in these situations. The genomic regions of interest are selected and enriched previous to next-generation sequencing. A frequently used application for the enrichment of the target sequences is based on hybridization with pre-designed probes, either on microarrays or in solution. The hybridized molecules are then captured (eluted from the microarrays or pulled-down from the solution, respectively), amplified and sequenced.

Besides quality control of the sequencing data, it is also crucial to assess whether the capture had been successful, i.e. if most of the sequenced reads actually fall on the target, if the targeted bases reach sufficient coverage, and so
This package provides functionalities to address this issue. Quality measures comprise specificity and sensitivity of the capture, enrichment, per-target read coverage, coverage uniformity and reproducibility, and read duplicate analysis. The coverage can further be examined for its relation to target region length and GC content of the hybridization probes. The analyses can be based on either single reads or read pairs in case of paired-end sequence data. Results are given as values (e.g. enrichment), tables (e.g. per-target coverage), and several diagnostic plots. The package makes use of data structures and methods from the \texttt{Ranges} package, which makes dealing with large sequence data feasible.

\textit{TEQC} does not include general sequencing data quality control (e.g. Phred quality plots), neither tools for sequence alignment. It also does not provide functionalities for follow-up analysis like SNP detection.

## 2 Automated html Reports

The fastest way to run \textit{TEQC} quality analysis is to launch the html report generation. The main arguments for \texttt{TEQCreport} are tables containing positions of sequenced reads and of genomic targets, or information about how to read the respective data files. There are several other options for customization of the report. In especially, you can choose whether or not to produce certain figures or coverage wiggle files and whether the data should be treated as single- or paired-end data. Calculation-intensive R objects can also be saved as workspace, for further usage. With option \texttt{destDir} you can specify the output folder, where all results will go and where you will find the index.html page of the html report. The parameters \texttt{sampleName}, \texttt{targetsName} and \texttt{referenceName} simply allow to include information about the sample and experiment in the report.

\begin{verbatim}
> library(TEQC)
>
exptPath <- system.file("extdata", package = "TEQC")
> readsfile <- file.path(exptPath, "ExampleSet_Reads.bed")
> targetsfile <- file.path(exptPath, "ExampleSet_Targets.bed")

> TEQCreport(sampleName = "Test Sample",
+ targetsName = "Human Exome",
+ referenceName = "Human Genome",
+ destDir = "report",
+ reads = get.reads(readsfile, skip = 0, idcol = 4),
+ targets = get.targets(targetsfile, skip = 0),
+ genome = "hg19")
\end{verbatim}

The function \texttt{TEQCreport} provides a quality report for only one sample at a time. Usually research projects include several samples, and it might be of interest to compare enrichment quality among all of them. Since it is cumbersome to check the \textit{TEQC} reports one by one, we provide the function \texttt{multiTEQCreport}, which collects results from previously created single-sample reports and summarizes them in a new html report. While the creation of single-sample reports is time and memory intensive, \texttt{multiTEQCreport} finishes quickly, since no heavy calculations have to be done anymore. The main input to this function are the
paths to the respective TEQC report output folders and the sample names that shall be used in tables and plots. The results can be browsed via the index.html file in a new destDir output directory.

As an example, let's assume that we have already present TEQC reports for two samples in folders report and report2, respectively. Then the multiple sample report can be created by

```r
> multiTEQCreport(singleReportDirs = c("report", "report2"),
+ samplenames = c("Sample A","Sample B"),
+ projectName = "Test Project",
+ targetsName = "Human Exome",
+ referenceName = "Human Genome",
+ destDir = "multiTEQCreport")
```

For details about the different steps, options and results of a TEQC analysis, it is recommended to read also the following sections of this documentation.

3 Load Reads and Targets Data

The (minimum) input needed for the quality control analysis are two files

- A bed file containing the genomic positions (chromosome, start, end) of the targeted regions, one genomic range per line. The targets might be custom designed or commercial solutions, e.g., for the capture of the whole human exome. The file does not have to be sorted with respect to genomic position.

- A bed or BAM file containing the genomic positions (chromosome, start, end) of sequenced reads aligned to a reference genome, one genomic range per line. In case of paired-end data an additional column with the read pair ID is suggested. The bed file format is very general and hence the QC analysis is not limited to any sequencing platform or alignment tool. The file does not have to be sorted with respect to genomic position. The BAM file format is now quite standard in NGS analysis. The usage of bam files has the advantage that reading the data is much faster (using functionality of the ShortRead package) than with bed files.

The package includes a small example data set. First we load the target positions. The input targetsfile does not need to have a fixed format. Just three columns containing chromosome (as string, e.g., chr1), start and end position of each target are required. The options chrCol, startCol and endCol specify in which columns of the file the respective information is found. The output of get.targets is of class GRanges from the GenomicRanges package. Note that overlapping or adjacent targets are merged, such that the returned target regions are not-overlapping. Therefore, also a bed file containing information about the hybridization probes, which might be highly overlapping due to tiling, can be used as targetsfile.

```r
> library(TEQC)
> exptPath <- system.file("extdata", package = "TEQC")
```
> targets <- get.targets(targetsfile=paste(exptPath, "ExampleSet_Targats.bed", +
    sep="/"), chrcol = 1, startcol = 2, endcol = 3, skip = 0)

[1] "read 50 (non-overlapping) target regions"

> targets

GRanges object with 50 ranges and 0 metadata columns:

<table>
<thead>
<tr>
<th>seqnames</th>
<th>ranges</th>
<th>strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;Rle&gt;</td>
<td>&lt;IRanges&gt;</td>
<td>&lt;Rle&gt;</td>
</tr>
<tr>
<td>[1]</td>
<td>chr1 11158025-11158264</td>
<td>*</td>
</tr>
<tr>
<td>[2]</td>
<td>chr1 25870174-25870293</td>
<td>*</td>
</tr>
<tr>
<td>[3]</td>
<td>chr1 65656333-65656572</td>
<td>*</td>
</tr>
<tr>
<td>[4]</td>
<td>chr1 68611504-68611743</td>
<td>*</td>
</tr>
<tr>
<td>[5]</td>
<td>chr1 70225862-70226101</td>
<td>*</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>[46]</td>
<td>chr21 33641314-33641433</td>
<td>*</td>
</tr>
<tr>
<td>[47]</td>
<td>chr22 30738144-30738263</td>
<td>*</td>
</tr>
<tr>
<td>[48]</td>
<td>chr22 32630850-32631089</td>
<td>*</td>
</tr>
<tr>
<td>[49]</td>
<td>chrX  47086386-47086505</td>
<td>*</td>
</tr>
<tr>
<td>[50]</td>
<td>chrX  150891046-150891285</td>
<td>*</td>
</tr>
</tbody>
</table>

seqinfo: 21 sequences from an unspecified genome; no seqlengths

NOTE: We assume that genomic positions in bed files follow the 0-based start / 1-based end coordinate system as defined by UCSC [http://genome.ucsc.edu/FAQ/FAQformat]. In TEQC we need 1-based coordinates, so by default get.targets and get.reads (see later) shift all start positions forward by 1. If the coordinates in your files are already 1-based, set the parameter zero based to FALSE in order to avoid the shifting.

We might ask what fraction of the genome is targeted. In the function fraction.target the corresponding genome can be specified by the option genome. At the moment only hg18, hg19 and hg38 are available. The corresponding genome sizes are taken from [http://genomesekicile.ucsc.edu/index.php/Genome_size_statistics](http://genomesekicile.ucsc.edu/index.php/Genome_size_statistics) For any other case, you can specify the genome size manually with the option genomesize. In our little example the total targeted region and hence the fraction within the genome is very small.

> ft <- fraction.target(targets, genome = "hg38")

> ft

[1] 4.06539e-06

Next, we load the genomic positions of the aligned reads. Depending on the number of reads, this can be quite time and memory consuming with real data. The function get.reads is quite similar to get.targets. However, overlapping or identical reads are not merged. Furthermore, a column containing read identifiers can be specified with option idcol. This is essential in case of paired-end data, when you want quality statistics to be done (also) on read pairs rather than on single reads. In this case the ID has to be the identifier of the read pair (i.e. the same unique ID for both reads of the pair). Our example data
was derived by paired-end sequencing, so we keep the pair IDs in the resulting 
GRanges object. The genomic positions in our bedfile containing the reads are 
1-based, so we avoid coordinate shifting by setting zerobase to FALSE.

```r
> reads <- get.reads(paste(exptPath, "ExampleSet_Reads.bed", sep="/"),
+ chrcol = 1, startcol = 2, endcol = 3, idcol = 4, zerobased = F, skip = 0)
```

```r
[1] "read 19546 sequenced reads"

> reads

GRanges object with 19546 ranges and 0 metadata columns:

<table>
<thead>
<tr>
<th>seqnames</th>
<th>ranges</th>
<th>strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>1_16_7090_2464</td>
<td>chr1 13328-13381</td>
<td>*</td>
</tr>
<tr>
<td>1_16_7090_2464</td>
<td>chr1 13467-13520</td>
<td>*</td>
</tr>
<tr>
<td>1_99_1631_6326</td>
<td>chr1 1420325-1420378</td>
<td>*</td>
</tr>
<tr>
<td>1_99_1631_6326</td>
<td>chr1 1420402-1420455</td>
<td>*</td>
</tr>
<tr>
<td>1_5_14614_17275</td>
<td>chr1 2321365-2321418</td>
<td>*</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>1_87_3763_6007</td>
<td>chrY 3551666-3551719</td>
<td>*</td>
</tr>
<tr>
<td>1_63_1439_10606</td>
<td>chrY 10028334-10028387</td>
<td>*</td>
</tr>
<tr>
<td>1_63_1439_10606</td>
<td>chrY 10028434-10028487</td>
<td>*</td>
</tr>
<tr>
<td>1_105_17963_15521</td>
<td>chrY 28425424-28425477</td>
<td>*</td>
</tr>
<tr>
<td>1_105_17963_15521</td>
<td>chrY 28425590-28425643</td>
<td>*</td>
</tr>
</tbody>
</table>

seqinfo: 24 sequences from an unspecified genome; no seqlengths

Whenever BAM files are available, it is recommended to use those instead of bed files as input for get.reads, while setting parameter filetype to "bam". In this case, get.reads makes use of the ShortRead function scanBam, with flag options isUnmappedQuery = FALSE and RfunargisSecondaryAlignment = FALSE. Note that multiple matches per read on the genome are not supported. Only primary alignments are read from the BAM file. All other previously described options of get.reads are ignored, since genomic positions and read identifiers are taken automatically from the BAM file.

```r
> reads <- get.reads("myBAM.bam", filetype = "bam")
```

### Paired-end data

When reads are paired, in order to perform statistics on pairs rather than on single reads, the read pairs have to be matched together using function reads2pairs. To run the function can be quite time consuming, depending on the number of reads. The output is a GRanges object whose ranges start at the first base of the first read within a read pair and end at the last base of the respective second read. This is equivalent to the positions of the DNA molecule that was actually sequenced from both ends. The reads table might also contain single reads (i.e. whose corresponding partners did not align to the reference). In this case a list of two GRanges tables will be returned, the first one containing the original positions of the single reads without partners (table singleReads),
and the other one containing the merged pairs positions (table `readpairs`). The provided reads data might also contain cases where the two reads of a pair align to different chromosomes. Since for such read pairs a 'merging' does not make sense, they will be returned within the `singleReads` table. Further, for some pairs the respective reads might align very far apart within the same chromosome. If you wish to remove such reads, you can specify a value for option `max.distance`. Reads with a larger distance (from start position of first read to end position of second read) will be added to table `singleReads`.

```r
data <- read.table('path/to/your/data.txt', header = TRUE, row.names = 1)
```

You may decide to use for all further analyses only "valid pairs", i.e. to exclude those reads that ended up in the `singleReads` table (in case there were any). This can be done e.g. in the following way:

```r
> validPairs <- reads2pairs(data)
> validPairs
```

You may decide to use for all further analyses only "valid pairs", i.e. to exclude those reads that ended up in the `singleReads` table (in case there were any). This can be done e.g. in the following way:

```r
> validPairs <- reads2pairs(data)
> validPairs
```

The provided reads data might also contain cases where the two reads of a pair align to different chromosomes. Since for such read pairs a 'merging' does not make sense, they will be returned within the `singleReads` table. Further, for some pairs the respective reads might align very far apart within the same chromosome. If you wish to remove such reads, you can specify a value for option `max.distance`. Reads with a larger distance (from start position of first read to end position of second read) will be added to table `singleReads`.

```r
> validPairs <- reads2pairs(data)
> validPairs
```

You may decide to use for all further analyses only "valid pairs", i.e. to exclude those reads that ended up in the `singleReads` table (in case there were any). This can be done e.g. in the following way:

```r
> validPairs <- reads2pairs(data)
> validPairs
```

Again, only for the case of paired-end data, we can visualize the read pair insert sizes, i.e. the distances from the start of read 1 to the end of read 2, respectively. For the function `insert.size.hist` we need the output of the previous call to `reads2pairs`. In our example, the alignment was done in a way that reads were only kept if the two reads aligned at a maximum distance of 250 bases. Therefore, the resulting histogram is truncated at 250, see figure 1. Note that also average and median insert sizes displayed in the graph are based on the truncated data, and hence are not statistics for the true read pair distribution. The insert sizes for all read pairs can also be returned using option `returnInserts = TRUE`.

```r
> validPairs <- reads2pairs(data)
> validPairs
```

Again, only for the case of paired-end data, we can visualize the read pair insert sizes, i.e. the distances from the start of read 1 to the end of read 2, respectively. For the function `insert.size.hist` we need the output of the previous call to `reads2pairs`. In our example, the alignment was done in a way that reads were only kept if the two reads aligned at a maximum distance of 250 bases. Therefore, the resulting histogram is truncated at 250, see figure 1. Note that also average and median insert sizes displayed in the graph are based on the truncated data, and hence are not statistics for the true read pair distribution. The insert sizes for all read pairs can also be returned using option `returnInserts = TRUE`.

```r
> validPairs <- reads2pairs(data)
> validPairs
```
4 Specificity and Enrichment

One important component of quality control in target capture experiments is to check whether most of the sequenced reads actually fall on target regions. A barplot showing the numbers of reads aligning to each chromosome can give a first impression on that. When providing the function `chrom.barplot` only with the reads table, the resulting barplot will show absolute counts. There is also the option to give both the reads and the targets table, which will show fractions of reads and targets, respectively, falling on each chromosome. For the reads, this is the fraction within the total number of reads (since reads are usually expected to have all the same length). In contrast, for the targets, the fraction of targeted bases on each chromosome is calculated. Since targets might strongly vary in length it is reasonable to account for the actual target sizes instead of considering merely numbers of targets per chromosome. In this way you can compare directly if the amount of reads corresponds more or less to the amount of target on a certain chromosome (see figure 2).

```r
> chrom.barplot(reads, targets)
```

A measure for the capture specificity is the fraction of aligned reads that overlap with any target region. It can be calculated by function `fraction.reads.target`. The function has an option `mappingReads` that can be set to `TRUE` in order to retrieve a reduced `readGRanges` table containing only those reads overlapping...
In many of the functions within TEQC you can specify an "offset" that will enlarge every target on each side by the specified number of bases. Since usually the captured DNA molecules are longer than what is actually sequenced, it is expected to have many reads that do not overlap, but are close to the target. Considering e.g. the actual targets plus 100 bases on each side, we get a higher on-target fraction:

```r
> fraction.reads.target(reads, targets, Offset=100)
[1] 0.4853679
```

With the fraction of on-target reads and the fraction of the targeted region within the reference genome, we can calculate the enrichment

\[
enrichment = \frac{\text{# reads on target}}{\text{# aligned reads}} \times \frac{\text{target size}}{\text{genome size}}
\]

Since in our artificial example the total target size is unrealistically small, we achieve a huge enrichment.

```r
> fr / ft
[1] 95907.34
```

Instead of considering single reads, we could also calculate the fraction of read pairs that are on-target. A read pair is counted as on-target if at least one of its reads overlaps with a target region or, in case of small targets, if the first read lies "left" and the second read "right" of the target and hence the corresponding sequenced molecule covered the target completely. For the specificity calculation the same function can be used, just the input changes from the table containing all reads to the table created above by \texttt{reads2pairs}. 

---

**Figure 2:** Fractions of reads (green) and target (orange) per chromosome.
5 Coverage

Besides high capture specificity, it is of course important to check the read coverage within target regions, since it is crucial for follow-up analyses. The function `coverage.target` calculates read coverage for each base that is sequenced and/or located in a target region. It returns a list with the average (list element `avgTargetCoverage`), standard deviation (`targetCoverageSD`) and main quantiles (`targetCoverageQuantiles`) of the coverage over all targeted bases. When option `perBase` is set to `TRUE`, the returned list additionally has the two elements `coverageAll` and `coverageTarget`. The former one is a `SimpleRleList` containing coverages for all bases present in the `reads` table, the latter one contains coverages for all targeted bases. For some TEQC functions we need either one or the other. When option `perTarget` is set to `TRUE`, the returned list has the additional element `targetCoverages`. This is the input `targets` `GRanges` table, now including as 'values' columns the average coverage (column `avgCoverage`) and standard deviation (column `coverageSD`) per target region. Coverage calculations might take a while, depending on the numbers of reads and targets.

```r
> Coverage <- coverage.target(reads, targets, perTarget=T, perBase=T)
> Coverage

$avgTargetCoverage
[1] 27.47927

$targetCoverageSD
[1] 23.68119

$targetCoverageQuantiles
 0%  25%  50%  75% 100%
   0    8   21   42  117

$targetCoverages
  GRanges object with 50 ranges and 2 metadata columns:
<table>
<thead>
<tr>
<th>names</th>
<th>ranges</th>
<th>strand</th>
<th>avgCoverage</th>
<th>coverageSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>chr1</td>
<td>11158025-11158264</td>
<td>*</td>
<td>44.1292</td>
<td>10.08893</td>
</tr>
<tr>
<td>chr1</td>
<td>25870174-25870293</td>
<td>*</td>
<td>0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>chr1</td>
<td>66566333-66566572</td>
<td>*</td>
<td>31.7583</td>
<td>6.93122</td>
</tr>
<tr>
<td>chr1</td>
<td>68611504-68611743</td>
<td>*</td>
<td>28.7958</td>
<td>13.48934</td>
</tr>
<tr>
<td>chr1</td>
<td>70226862-70226101</td>
<td>*</td>
<td>24.1667</td>
<td>8.04936</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>chr21</td>
<td>33641314-33641433</td>
<td>*</td>
<td>4.40000</td>
<td>1.53612</td>
</tr>
<tr>
<td>chr22</td>
<td>30738144-30738263</td>
<td>*</td>
<td>28.28333</td>
<td>4.44421</td>
</tr>
<tr>
<td>chr22</td>
<td>32630850-32631089</td>
<td>*</td>
<td>7.56667</td>
<td>3.27852</td>
</tr>
<tr>
<td>chrX</td>
<td>47086386-47086505</td>
<td>*</td>
<td>14.59167</td>
<td>2.82693</td>
</tr>
</tbody>
</table>
```

> fraction.reads.target(readpairs, targets)

[1] 0.4934002
seqinfo: 21 sequences from an unspecified genome; no seqlengths

coverageAll
RleList of length 24
chr1
integer-Rle of length 248737162 with 3785 runs
  Lengths: 13327 54 85 54 ... 185876 54 130 54
  Values : 0 1 0 1 ... 0 1 0 1

chr2
integer-Rle of length 242418478 with 2596 runs
  Lengths: 103676 54 53 54 ... 215014 54 50 54
  Values : 0 1 0 1 ... 0 1 0 1

chr3
integer-Rle of length 197703049 with 1774 runs
  Lengths: 439910 54 37 54 ... 84392 54 141 54
  Values : 0 1 0 1 ... 0 1 0 1

chr4
integer-Rle of length 190962876 with 1511 runs
  Lengths: 1633781 54 111 54 ... 1532357 54 66 54
  Values : 0 1 0 1 ... 0 1 0 1

chr5
integer-Rle of length 180661803 with 1691 runs
  Lengths: 225819 54 92 54 ... 178562 54 13 54
  Values : 0 1 0 1 ... 0 1 0 1

... <19 more elements>

coverageTarget
RleList of length 21
chr1
integer-Rle of length 2520 with 1079 runs
  Lengths: 1 1 1 1 2 2 1 2 2 1 2 2 1 1 1 2 2 1 2 2 1 1 1 1 1 1 1 ... 1 1 2 1 1 2 1 1 1 1
  Values : 23 22 21 23 24 23 24 21 22 21 22 1 ... 39 40 38 37 36 35 34 33 34 33

chr2
integer-Rle of length 960 with 587 runs
  Lengths: 1 2 1 2 1 1 1 1 1 1 ... 3 2 4 2 1 2 1 1 1 2
  Values : 16 15 16 17 16 18 17 18 16 15 14 ... 25 23 22 21 20 19 18 19 18 17

chr3
integer-Rle of length 1647 with 516 runs
  Lengths: 2 2 1 2 2 2 1 1 2 2 1 ... 2 4 1 4 2 1 1 4 2 2
  Values : 31 33 32 31 32 31 30 29 33 31 32 ... 10 8 9 10 11 10 11 12 13 12
integer-Rle of length 840 with 337 runs
Lengths: 2 1 1 1 5 1 2 1 2 1 1 ... 1 3 2 4 3 1 3 2 1 3
Values : 42 43 42 44 43 41 40 39 38 36 35 ... 22 21 22 25 26 25 26 25 24 23

integer-Rle of length 240 with 157 runs
Lengths: 3 1 1 1 3 1 1 2 1 2 1 ... 2 3 1 2 1 3 2 1 1 3
Values : 55 60 59 58 56 58 60 63 64 62 ... 41 40 41 42 41 43 42 46 44 45
...
<16 more elements>

> targets2 <- Coverage$targetCoverages

Note that coverage should not be calculated using the output of function reads2pairs in place of reads, since the genomic ranges in that table span the whole region from the first to the second read of a pair. However, for the respective read insert there is no actual coverage, because only the ends of the original fragment were sequenced.

A different measure for the per-target coverage would be to count the numbers of reads overlapping with each target. This can be calculated by the function readsPerTarget. It returns again the input targets table while adding a 'values' column that gives the respective numbers of reads per target. If we provide the table targets2 from above, we will get both the average per-target coverages and the numbers of target-overlapping reads in the same table. In order to speed things up, instead of all reads we could also provide the mappingReads output of function fraction.reads.target, since for counting the reads per target it is enough to look at the reads from which we already know they are on-target. Just make sure that the Offset option was set the same in both fraction.reads.target and readsPerTarget.

> targets2 <- readsPerTarget(reads, targets2)
> targets2

GRanges object with 50 ranges and 3 metadata columns:

<table>
<thead>
<tr>
<th>seqnames</th>
<th>ranges</th>
<th>strand</th>
<th>avgCoverage</th>
<th>coverageSD</th>
<th>nReads</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;Rle&gt;</td>
<td>&lt;IRanges&gt;</td>
<td>&lt;Rle&gt;</td>
<td>&lt;numeric&gt;</td>
<td>&lt;numeric&gt;</td>
<td>&lt;numeric&gt;</td>
</tr>
<tr>
<td>[1]</td>
<td>chr1 11158025-11158264</td>
<td>*</td>
<td>44.1292</td>
<td>10.08893</td>
<td>231</td>
</tr>
<tr>
<td>[2]</td>
<td>chr1 26870174-26870293</td>
<td>*</td>
<td>0.0000</td>
<td>0.00000</td>
<td>0</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>[46]</td>
<td>chr21 33641314-33641433</td>
<td>*</td>
<td>4.40000</td>
<td>1.53612</td>
<td>15</td>
</tr>
<tr>
<td>[47]</td>
<td>chr22 30738144-30738263</td>
<td>*</td>
<td>28.2833</td>
<td>4.44421</td>
<td>85</td>
</tr>
<tr>
<td>[46]</td>
<td>chr22 32630860-32631089</td>
<td>*</td>
<td>7.56667</td>
<td>3.27852</td>
<td>38</td>
</tr>
<tr>
<td>[49]</td>
<td>chrX 47086386-47086605</td>
<td>*</td>
<td>14.5617</td>
<td>2.82693</td>
<td>46</td>
</tr>
<tr>
<td>[50]</td>
<td>chrX 150891046-150891285</td>
<td>*</td>
<td>10.0083</td>
<td>2.72443</td>
<td>51</td>
</tr>
</tbody>
</table>
The resulting \textit{GRanges} table can be converted to a data frame, and as such easily be written to a file, e.g. by

\begin{verbatim}
> write.table(as.data.frame(targets2), file="target_coverage.txt", sep="\t", row.names=F, quote=F)
\end{verbatim}

Talking about coverage, it is interesting to ask which fraction of target bases reach a coverage of at least \textit{k} (some value relevant for further analyses, e.g. SNP calling) or which fraction of target bases is covered at all by any read (sensitivity of the capture). The function \texttt{covered.k} calculates such fractions based on the \texttt{coverageTarget} output of \texttt{coverage.target}. Option \texttt{k} specifies the values for which to calculate the fraction of bases achieving the respective coverage.

\begin{verbatim}
> covered.k(Coverage$coverageTarget, k=c(1, 5, 10))
\end{verbatim}

\begin{verbatim}
1 5 10
0.9520963 0.8444087 0.7033801
\end{verbatim}

With \texttt{coverage.hist} we can visualize the coverage distribution, see figure 3. A line is added to the histogram that shows the cumulative fraction of target bases with a coverage of at least the corresponding x-axis value. The line represents the results of \texttt{covered.k} for all possible values of \texttt{k}. Additionally, you can highlight with dashed lines the base fraction achieving a coverage of at least a certain value by defining the option \texttt{covthreshold}.

\begin{verbatim}
> coverage.hist(Coverage$coverageTarget, covthreshold=8)
\end{verbatim}

A similar graph is the coverage uniformity plot, see figure 4. It corresponds more or less to the cumulative line in the coverage histogram. However, \texttt{coverage.uniformity} calculates normalized coverages, i.e. the per-base coverages divided by the average coverage over all target bases. Normalized coverages are not dependent on the absolute quantity of sequenced reads and are hence better comparable between different samples or even different experiments. By default, the x-axis in the figure is truncated at 1, which corresponds to the average normalized coverage. The steeper the curve is falling, the less uniform is the coverage.

\begin{verbatim}
> coverage.uniformity(Coverage)
\end{verbatim}

There are more graphical functions concerning read coverage. For example you might be interested in whether large targets are covered by more reads, as expected, since for larger target regions there should also be more hybridization capture probes ("baits"). Or you might ask whether quite small targets have worse coverage, because the bait tiling might not be as good as for larger targets. Those questions can be addressed by the function \texttt{coverage.targetlength.plot}. As input a \textit{GRanges} targets table has to be given that contains the relevant information in the ‘values’ column(s). The graphs are useful for targets of very different lengths. In our example, where all the targets are rather small, the
Figure 3: On-target coverage histogram. The orange line shows the cumulative fraction of target bases (right y-axis) with a read coverage of at least \(x\). The dashed lines highlight the fraction of target bases covered by at least 8 reads.

Another thing to check is the dependency between coverage and GC content of the hybridization capture probes ("baits"). For calculating the GC contents the bait sequences are needed. A file has to be created beforehand that contains the positions as well as the sequences of all baits. The file is loaded by function get.baits which is similar to get.targets, with the difference that overlapping or adjacent baits are not merged, and that a column seqcol has to be specified that holds the bait sequences. Like get.targets and get.reads, also get.baits by default converts the bait start positions from 0-based to 1-based coordinates. If the positions given in your baitsfile are already 1-based, set option zeroBased to FALSE.

```r
> baitsfile <- paste(exptPath, "ExampleSet_Baits.txt", sep="/"")
> baits <- get.baits(baitsfile, chrcol=3, startcol=4, endcol=5, seqcol=2)
```
Figure 4: Fraction of targeted bases (y-axis) achieving a normalized coverage of at least \( x \). Dashed lines indicate the fractions of bases achieving at least the average \( (= 1) \) or at least half the average coverage \( (= 0.5) \).

Figure 5: Scatter plots and smoothing splines of number of reads per target (left panel) or average coverage per target (right panel) versus respective target length.

[1] "read 108 hybridization probes"

With the baits \textit{Ranges} table and the \texttt{coverageAll} output of \texttt{coverage.target} the (normalized) coverage versus GC content plot can be created. This can take quite some time, since GC content and average (normalized) coverage have to
be calculated for every bait. The bait coverages can be returned by setting option *returnBaitValues = TRUE*. You would expect the added smoothing spline to have an inverse U-shape, with a peak in coverage for baits with GC content around 40-50%. In our small example there are not enough baits with low GC content to encounter the expected shape, see figure 6.

> coverage.GC(Coverage$coverageAll, baits, pch=16, cex=1.5)

Figure 6: Scatter plot and smoothing spline of normalized average coverage per hybridization probe versus GC content of the respective bait.

There is also a function `coverage.plot` to visualize per-base coverages along chromosomal positions. The input has to be the `coverageAll` output of function `coverage.target`, since also coverages of off-target bases are needed. The positions of target regions, potentially extended on both sides, can be highlighted as well by specifying options `targets` and `Offset` (see figure 7).

> coverage.plot(Coverage$coverageAll, targets, Offset=100, chr="chr1", Start=11157524, End=11158764)

Of course, coverages can also be visualized by genome browsers. We provide the function `make.wigfiles` to create wiggle files that can then be uploaded e.g. to the UCSC genome browser. You can make wiggle files for all chromosomes on which there are reads or just for some selected chromosome(s) by specifying option `chroms`. With option `filename` the name and location where to save
the files can be manipulated. Base positions are given as 1-based (i.e. the first base on a chromosome has position 1), as defined for wiggle files by UCSC (http://genome.ucsc.edu/goldenPath/help/wiggle.html).

> make.wigfiles (Coverage$coverageAll)

### 6 Read Duplicates

A crucial issue in target capture experiments is read duplication. Usually, read duplicates, i.e. reads that have exact same start and end positions, are removed before follow-up analysis of sequencing data, since they are supposed to be PCR artifacts. However, here we expect a probably substantial amount of "real" read duplication due to the enrichment process. "Real" read duplicates would be derived from actually separate input DNA molecules that by chance were fragmented at the same position. The duplicates.barplot shows which fraction of reads / read pairs is present in the data in what number of copies. Read multiplicity proportions are calculated and shown separately for on- and off-target reads / read pairs. Therefore, the plot gives an impression about the amount of "real" duplication (expected mostly in the target regions) versus artificial duplication (expected both on- and off-target). With option returnDups = TRUE the absolute numbers (given on top of the bars, in millions) and percentages (bar heights) can be returned.
> duplicates.barplot(reads, targets)

Figure 8: Duplicates barplot treating data as single-end. It shows fractions of on- and off-target reads, respectively, that are unique, that are there in two copies, three copies, etc. (x-axis). The numbers on top of the bars are absolute counts in millions.

In figure 8 we see, firstly, that for some reads there are quite a lot of identical copies (x-axis up to 10). Secondly, the percentage of reads with multiple copies is much higher within on-target reads than within off-target reads (red bars are much higher than blue ones for \( x > 1 \)). This suggest, as mentioned before, that there might be substantial amount of "real" read duplication.

In the case of paired-end data, the position information of both reads of a pair can be used. Reads only have to be considered duplicated if the positions of both reads are found again in another pair. We can use duplicates.barplot with the table readpairs we created before to make the graph for read pairs instead of single reads. For read pairs the extent of duplication is by far not as high as for single reads, see figure 9.

> duplicates.barplot(readpairs, targets, ylab="Fraction of read pairs")

Unfortunately, it is not possible to distinguish the artefactual duplicates from the naturally occurring ones. For that reason it might still be recommendable to remove duplication before further analysis by keeping each duplicated read position only once. (Since here we just deal with genomic positions and not with
the actual reads that might differ in sequence and quality, it does not matter which copy of a duplicated read to keep.) You might also decide to do all the QC analysis on the "collapsed" data. Either a bed file is provided that includes only unique read positions, or the tables are collapsed within R, e.g. by the following code.

```r
> reads.collapsed <- unique(reads)
```

In the case of paired-end data, as said before, reads only have to be considered duplicated if the positions of both reads are found again in another pair. Removing duplicates can be done like follows, selecting non-duplicated reads from the readpairs table (and in case of duplication just keep one of the respective reads) and extracting those from the original read table.

```r
> ID.nondups <- names(unique(readpairs))
> reads.collapsed.pairs <- reads[names(reads) %in% ID.nondups,,drop=T]
```

In single-end data it is probable to lose a large number of reads by removing duplicates. When treating our example data like single-end sequences, we are left with only 16417 out of originally 19546 reads. Hence, the actual coverage could decrease dramatically.

```r
> coverage.target(reads.collapsed, targets, perBase=F, perTarget=F)
```
$avgTargetCoverage
[1] 17.43535

$targetCoverageSD
[1] 11.7878

$targetCoverageQuantiles
 0%  25%  50%  75% 100%
 0   7  16  27  46

Before removing duplicates we calculated a coverage of 27.5.
When using position information of both reads per pair, much less reads are
lost, e.g. in the example we can keep 18714 reads. Therefore, the coverage will
not decrease that much.

> coverage.target(reads.collapsed.pairs, targets, perBase=F, perTarget=F)
$avgTargetCoverage
[1] 25.09604

$targetCoverageSD
[1] 21.22714

$targetCoverageQuantiles
 0%  25%  50%  75% 100%
 0   7  20  38 110

7 Reproducibility

For any new technology, reproducibility is an important issue. Here we base
the reproducibility check on per-target-base coverages. Especially for technical
replicates we should yield similar results. But the following graphs might also
be useful to ensure homogeneity across biological replicates.

To give an example, we create an artificial new sample by removing randomly
10% of the reads from our data.

> r <- sample(length(reads), 0.1 * length(reads))
> reads2 <- reads[-r,,drop=T]
> Coverage2 <- coverage.target(reads2, targets, perBase=T)

With function coverage.density the coverage densities of several samples
can be compared. With option normalized you can choose whether to plot origi-
nal or normalized coverages. When plotting original values in the example, it is
obvious that the second sample does not reach as high coverage for many bases
as the first one, see figure 10 right panel. In contrast, normalized coverages
are not dependent on the total amount of reads, and we observe a very similar
coverage distribution for both samples (left panel), as expected since we just
removed some reads randomly.

> covlist <- list(Coverage, Coverage2)
> par(mfrow=c(1,2))
> coverage.density(covlist)
> coverage.density(covlist, normalized=F)

Figure 10: Target coverage densities of two samples, using normalized (left) or original (right) coverages.

Also the coverage uniformity and coverage along chromosome plots shown above (figures 4 and 7) can be produced for several samples within the same graph. The functions can be called repeatedly, while specifying option `add=TRUE` (see figures 11 and 12). As for the densities, the uniformity is almost identical for the two samples since normalized coverage values are used for this plot.

> coverage.uniformity(Coverage, addlines=F)
> coverage.uniformity(Coverage2, addlines=F, add=T, col="blue", lty=2)

> coverage.plot(Coverage$coverageAll, targets, Offset=100, chr="chr1", Start=11157524, End=11158764)
> coverage.plot(Coverage2$coverageAll, add=T, col=line=2, chr="chr1", Start=11157524, End=11158764)

With function `coverage.correlation` we can produce scatterplots between coverage values of pairs of replicate samples. Since a scatterplot for all per-target-base coverages would be huge (e.g. as pdf graph), and moreover the difference between one million or ten million points in the graphic might not be visible at all, just some fraction of randomly selected values is displayed. The amount of points to plot can be controlled by option `plotfrac`. By default, 0.1% of all targeted bases are taken into account. In the example we set the fraction to 10%, see figure 13. Scatterplots are shown in the style of a pairs plot. In the lower panels the corresponding Pearson correlation coefficients are shown. Correlation calculations are always based on all coverage values, even if `plotfrac < 1` is chosen. Like in `coverage.density`, by option `normalized` it can be chosen whether to plot normalized or original coverage values.
> coverage.correlation(covlist, plotfrac=0.1, cex.pch=4)

8 Acknowledgement

The example data used in this manual was taken from an exome sequencing data set of Raquel Rabionet and Xavier Estivill.

9 Session Information

> toLatex(sessionInfo())

- R version 4.3.0 RC (2023-04-13 r84269), x86_64-pc-linux-gnu
- Locale: LC_CTYPE=en_US.UTF-8, LC_NUMERIC=C, LC_TIME=en_GB,
  LC_COLLATE=C, LC_MONETARY=en_US.UTF-8, LC_MESSAGES=en_US.UTF-8,
  LC_PAPER=en_US.UTF-8, LC_NAME=C, LC_ADDRESS=C, LC_TELEPHONE=C,
  LC_MEASUREMENT=en_US.UTF-8, LC_IDENTIFICATION=C
- Time zone: America/New_York
- TZcode source: system (glibc)
- Running under: Ubuntu 22.04.2 LTS

Figure 11: Target coverage uniformity of two samples.
Figure 12: Coverage along chromosome plot for two samples.

- Matrix products: default
- BLAS: `/home/biocbuild/bbs-3.17-bioc/R/lib/libRblas.so`
- LAPACK: `/usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.10.0`
- Base packages: `base, datasets, grDevices, graphics, methods, stats, stats4, utils`
- Other packages: `BioCGenerics 0.46.0, Biostrings 2.68.0, GenomeInfoDb 1.36.0, GenomicRanges 1.52.0, IRanges 2.34.0, Rsamtools 2.16.0, S4Vectors 0.38.0, TEQC 4.22.0, XVector 0.40.0, lmarkdown 1.3.2.1`
- Loaded via a namespace (and not attached): `Biobase 2.60.0, BioCParallel 1.34.0, GenomeInfoDbData 1.2.10, RCurl 1.98-1.12, bitops 1.0-7, codetools 0.2-19, compiler 4.3.0, crayon 1.5.2, parallel 4.3.0, tools 4.3.0, zlibbioc 1.46.0`

10 References

Figure 13. Correlation plots of normalized target coverage values of two samples.


