

R453Plus1Toolbox

A package for importing and analyzing data from
Roche's Genome Sequencer System

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1 Introduction

The R453Plus1 Toolbox comprises useful functions for the analysis of data generated by Roche's 454 sequencing platform. It adds functions for quality assurance as well as for annotation and visualization of detected variants, complementing the software tools shipped by Roche with their product. Further, a pipeline for the detection of structural variants is provided.

```
> library(R453Plus1Toolbox)
```

2 Analysis of PCR amplicon projects

This section deals with the analysis of projects investigating massively parallel data generated from specifically designed PCR products.

2.1 Importing a Roche Amplicon Variant Analyzer project

The function `AVASet` imports data from Roche's Amplicon Variant Analyzer (AVA). This can be done in three ways, depending on the version of your AVA software:

2.1.1 Import from AVA without AVA-CLI (version 2.5 and lower)

For projects created with the AVA software version ≤ 2.5 , `AVASet` expects only a `dirname` pointing to the project data, i.e. a directory that contains the following files and subdirectories:

- "Amplicons/ProjectDef/ampliconsProject.txt"
- "Amplicons/Results/Variants/currentVariantDefs.txt"
- "Amplicons/Results/Variants"
- "Amplicons/Results/Align"

There is an example project "AVASet" included in the *R453Plus1Toolbox* installation directory:

```
> projectDir = system.file("extdata", "AVASet", package = "R453Plus1Toolbox")
> avaSet = AVASet(dirname=projectDir)
```

2.1.2 Import from AVA with AVA-CLI

The function `AVASet` can directly access the AVA Command Line Interface (AVA-CLI) from within R. If the AVA software is installed on the same machine that runs R, the easiest way to import a project is to specify the project

directory with `dirname` and the path to the binaries in the AVA software's installation directory with `avaBin`. It is usually the directory "bin" containing the AVA-CLI command interpreter "doAmplicon".

Let's say the AVA software was installed to the directory "/home/User/AVA". Then, the function call looks like:

```
> projectDir = "My/AVA/Project"
> avaSet = AVASet(dirname=projectDir, avaBin="/home/User/AVA/bin")
```

2.1.3 Import from AVA with projects exported via AVA-CLI

If the AVA software is not installed on the same machine that runs R, all data must be exported manually using AVA-CLI. It can be accessed via the command line interpreter "doAmplicon" from the AVA software's installation directory. Within the AVA-CLI, load your project with the command "open". `AVASet` expects five files (variant information is optional):

AVASet argument	AVA-CLI command	Description
<code>file_sample</code>	list sample -outputFile sample.csv	Table with sample names and annotations
<code>file_amp</code>	list amplicon -outputFile amp.csv	Table with primer sequences, positions and annotations
<code>file_reference</code>	list reference -outputFile reference.csv	Reference sequences
<code>file_variant</code>	list variant -outputFile variant.csv	Detected variants (if available)
<code>file_variantHits</code>	report variantHits -outputFile variantHits.csv	Variant hits for all samples (if available)

Table 1: `AVASet` function arguments for loading projects exported via AVA-CLI.

Note, that all exported tables are expected to be in csv-format.

There is an example project "AVASet_doAmplicon" included in the *R453Plus1Toolbox* installation directory:

```
> projectDir = system.file("extdata", "AVASet_doAmplicon", package="R453Plus1Toolbox")
> avaSetExample = AVASet(dirname=projectDir, file_sample="sample.csv",
  file_amp="amp.csv", file_reference="reference.csv", file_variant="variant.csv",
  file_variantHits="variantHits.csv")
```

`AVASet` searches the specified `dirname` for the exported csv-files. `file_variant` `file_variantHits` can be omitted if no variant information is available for the project.

2.2 The AVASet class

The `AVASet` class defines a container to store data imported from projects conducted with Roche's AVA software. It extends the *Biobase eSet* to store all relevant information.

```

> avaSet

AVASet (storageMode: list)
assayData: 259 features, 6 samples
  element names: variantForwCount, totalForwCount, variantRevCount, totalRevCount
protocolData: none
phenoData
  sampleNames: Sample_1 Sample_2 ... Sample_6 (6
    total)
  varLabels: SampleID MID1 ... Annotation (7 total)
  varMetadata: labelDescription
featureData
  featureNames: C1438 C369 ... C763 (259 total)
  fvarLabels: name canonicalPattern ...
    referenceBases (7 total)
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation:
An object of class 'AnnotatedDataFrame'
  rowNames: TET2_E11.04 TET2_E06 TET2_E11.03 TET2_E04
  varLabels: ampID primer1 ... targetEnd (6 total)
  varMetadata: labelDescription
class: AlignedRead
length: 4 reads; width: 339..346 cycles
chromosome: NA NA NA NA
position: 1 1 1 1
strand: NA NA NA NA
alignQuality: NumericQuality
alignData varLabels: name refSeqID gene

```

An object of class *AVASet* consists of three main components:

1. Variants:

The variants part stores data about the found variants and is accessible by the functions `assayData`, `featureData` and `phenoData` known from Biobase eSet.

The `assayData` slot contains four matrices with variants as rows and samples as columns:

- `variantForwCount`: Matrix containing the number of reads with the respective variant in forward direction.
- `variantRevCount`: Matrix containing the number of reads with the respective variant in reverse direction.
- `totalForwCount`: Matrix containing the total coverage for every variant location in forward direction.

- `totalRevCount`: Matrix containing the total coverage for every variant location in reverse direction.

```
> assayData(avaSet)$totalForwCount[1:3, ]
```

	Sample_1	Sample_2	Sample_3	Sample_4	Sample_5	Sample_6
C1438	119	1516	137	1729	1288	140
C369	267	1152	195	1518	1016	190
C595	258	1805	230	1885	1775	221

The `featureData` slot provides additional information on the variants. `fData` returns a data frame with variants as rows and the following columns:

- `name/canonicalPattern`: Short identifiers of a variant including the position and the bases changed.
- `referenceSeq`: Gives the identifier of the reference sequence (see below).
- `start/end`: The position of the variant relative to the reference sequence.
- `variantBase/referenceBases`: The bases changed in the variant.

```
> fData(avaSet)[1:3, ]
```

	name	canonicalPattern	referenceSeqID	start	end
C1438	303:T/C	s(303,C)	I37	303	303
C369	309:T/C	s(309,C)	I36	309	309
C595	108:T/C	s(108,C)	I40	108	108

	variantBase	referenceBases
C1438	C	T
C369	C	T
C595	C	T

The `phenoData` slot provides sample-IDs, multiplexer IDs (MID1, MID2), the pico titer plate (PTP) accession number, the lane, the read group and additional textual annotation for each sample. Most of these informations are imported directly from Roche's software.

```
> pData(avaSet)
```

	SampleID	MID1	MID2	PTP_AccNum	Lane	ReadGroup
Sample_1	I9646	Mid3	Mid3	GGSFDBH	07	
Sample_2	I116	Mid1	Mid1	GA0582C	01	
Sample_3	I9644	Mid1	Mid1	GGSFDBH	07	
Sample_4	I118	Mid3	Mid3	GA0582C	01	
Sample_5	I117	Mid2	Mid2	GA0582C	01	
Sample_6	I9645	Mid2	Mid2	GGSFDBH	07	

Annotation

```

Sample_1
Sample_2
Sample_3
Sample_4
Sample_5
Sample_6

```

2. Amplicons:

This part stores information about the used amplicons and is accessible by the functions `assayDataAmp` and `fDataAmp`.

The slot `assayDataAmp` contains two matrices with amplicons as rows and samples as columns:

- `forwCount`: Matrix containing the number of reads for each amplicon and each sample in forward direction.
- `revCount`: Matrix containing the number of reads for each amplicon and each sample in reverse direction.

```
> assayDataAmp(avaSet)$forwCount
```

	Sample_1	Sample_2	Sample_3	Sample_4	Sample_5
TET2_E11.04	119	1516	137	1729	1288
TET2_E06	248	400	224	478	339
TET2_E11.03	267	1152	195	1518	1016
TET2_E04	258	1805	230	1885	1775
	Sample_6				
TET2_E11.04	140				
TET2_E06	204				
TET2_E11.03	190				
TET2_E04	221				

The slot `featureDataAmp` contains an `AnnotatedDataFrame` with additional information on each amplicon:

- `ampID`: The identifier of the current amplicon.
- `primer1`, `primer2`: The primer sequences for each amplicon.
- `referenceSeqID`: The identifier of the reference sequence (see below).
- `targetStart/targetEnd`: The coordinates of the target region.

```
> fDataAmp(avaSet)
```

	ampID	primer1
TET2_E11.04	I90	CATTACCTTCTCACATAATCCA
TET2_E06	I81	TGCAAGTGACCCTTGTTTGG
TET2_E11.03	I89	GCTCAGTCTACCACCCATCC
TET2_E04	I79	GGGGTTAAGCTTTGTGGATG

	primer2	referenceSeqID
TET2_E11.04	GAATTGACCCATGAGTTGGAG	I37
TET2_E06	AACCAAAGATTGGGCTTTC	I42
TET2_E11.03	AGATGCAGGGCATGAAGAGA	I36
TET2_E04	TTGTGACTCTCTGGTGAATAGCA	I40

	targetStart	targetEnd
TET2_E11.04	24	325
TET2_E06	21	321
TET2_E11.03	21	319
TET2_E04	21	322

As both refer to the same samples, the variants phenoData slot is used for amplicons as well.

3. Reference sequences:

This part stores data about the reference sequences the amplicons were selected from. All information is stored into an object of class *AlignedRead*. The reads are accessible via `sread`. To retrieve additional information from Ensembl about the chromosome, the position and the strand of each reference sequence run function `alignShortReads` (see section 2.6 for details).

```
> library(ShortRead)
> referenceSequences(avaSet)

class: AlignedRead
length: 4 reads; width: 339..346 cycles
chromosome: NA NA NA NA
position: 1 1 1 1
strand: NA NA NA NA
alignQuality: NumericQuality
alignData varLabels: name refSeqID gene

> sread(referenceSequences(avaSet))

A DNASTringSet instance of length 4
  width seq          names
[1]  345 GGGGTTAAGCTTT...CAGAGAGTCACAA I40
[2]  346 CATTACCTTCTC...CATGGGTCAATTC I37
[3]  339 GCTCAGTCTACCA...ATGCCCTGCATCT I36
[4]  341 TGCAAGTGACCCT...CCAATCTTTGGTT I42
```

The following table sums up the available slots and accessor functions:

Function/Slot	Description
<code>assayData</code>	Contains the number of reads and the total coverage for every variant and each sample in forward and reverse direction.
<code>fData/featureData</code>	Contains information about the type, position and reference of each variant.
<code>pData/phenoData</code>	Contains sample-IDs, multiplexer IDs (MID1, MID2), the pico titer plate (PTP) accession number, the lane, the read group and additional textual annotation for each sample.
<code>assayDataAmp</code>	Contains the number of reads for every amplicon and each sample in forward/reverse direction.
<code>fDataAmp/featureDataAmp</code>	Contains the primer sequences, reference sequence and the coordinates of the target region for each amplicon.
<code>referenceSequences</code>	Contains the reference sequences for the amplicons together with additional annotations.

Table 2: AVASet contents and accessor functions.

2.3 Subsetting an AVASet

A subset of an *AVASet* object can be generated using the common `[]`-notation:

```
> avaSubSet = avaSet[1:10, "Sample_1"]
```

The first dimension refers to the variants and the second dimension to the samples, so an *AVASet* with ten variants and one sample is returned.

This is a short and to some extend equivalent version of the function `subset`, which expects a `subset` argument and the respective `dimension` (either "variants", "samples" or "amplicons"):

```
> avaSubSet = subset(avaSet, subset=1:10, dimension="variants")
```

The following is equivalent to the `[]`-example above:

```
> avaSubSet = subset(subset(avaSet, subset=1:10, dimension="variants"), subset="Sample_1", dimension="samples")
```

In contrast to the `[]`-Notation, the function `subset` allows further subsetting by amplicons:

```
> avaSubSet = subset(avaSet, subset=c("TET2_E11.04", "TET2_E06"), dimension="amplicons")
```

When subsetting by amplicons all variants referring to amplicons that are not in the subset will be excluded.

2.4 Setting filters on an AVASet

Another way of generating a subset of an *AVASet* object is filtering only those variants, whose coverage (in percent) in forward and reverse direction respectively is higher than a given `filter` value in at least one sample. Here, the coverage is defined as the percentual amount of the reads with the given variant on the number of all reads covering the variant's position.

The function `setVariantFilter` returns an updated *AVASet* object that meets the given requirements:

```
> avaSetFiltered1 = setVariantFilter(avaSet, filter=0.05)
```

The above example returns an *AVASet*, which only contains variants whose coverage is greater than 5% in at least one sample.

Passing a vector of two `filter` values applies filtering according to forward and reverse read direction separately:

```
> avaSetFiltered2 = setVariantFilter(avaSet, filter=c(0.1, 0.05))
```

In fact, when filtering an *AVASet*, the whole object is still available. The filter only affects the output given by accessor functions like `fData`, `featureData` and `assayData`.

The process can be reversed and the filter value(s) can be reset to zero by calling

```
> avaSet = setVariantFilter(avaSetFiltered1, filter=0)
```

or simply

```
> avaSet = setVariantFilter(avaSetFiltered2)
```

2.5 Variant coverage

The function `getVariantPercentages` displays the coverage of the variants for a given `direction` (either "forward", "reverse", or "both"):

```
> getVariantPercentages(avaSet, direction="both")[20:25, 1:4]
```

	Sample_1	Sample_2	Sample_3	Sample_4
C386	0.00000000	0.00000000	0.00000000	0.00000000
C1808	0.00000000	0.00000000	0.00000000	0.00000000
C1338	0.00000000	0.002405774	0.45720251	0.00000000
C1052	0.03202847	0.044400452	0.03076923	0.06076519
C818	0.00000000	0.003019628	0.00000000	0.00000000
C681	0.00000000	0.00000000	0.00000000	0.00000000

In the example above, `getVariantPercentages` is simply a short form of calculating

```
> (assayData(avaSet)[[1]] + assayData(avaSet)[[3]]) / (assayData(avaSet)[[2]] + assayData(avaSet)[[4]])
```

2.6 Annotations and Variant Reports

Before creating the variant and quality report, the reference sequences must be aligned against a reference genome and afterwards the variants have to be annotated.

The method `alignShortReads` aligns the reference sequences from an *AVASet* against a given reference genome. Only exact (no errors) and unique matches are returned. In the example below the hg19 assembly as provided by UCSC from package *BSgenome.Hsapiens.UCSC.hg19* is used as reference:

```
> library(BSgenome.Hsapiens.UCSC.hg19)
> seqNames = names(Hsapiens)[1:24]
> avaSet = alignShortReads(avaSet, bsGenome=Hsapiens,
  seqNames=seqNames, ensemblNotation=TRUE)
```

The function `annotateVariants` annotates genomic variants (mutations) given in a data frame or more likely an *AVASet*. Annotation includes affected genes, exons and codons. Resulting amino acid changes are returned as well as dbSNP identifiers if the mutation is already known. All information is fetched from Ensembl via *biomaRt* and returned in an object of class *AnnotatedVariants*. It is advisable to filter the *AVASet* (see section 2.4) prior to that since the annotation process is very time consuming for a large number (>500) of variants.

```
> avaSet = setVariantFilter(avaSet, filter=0.05)
> avaAnnot = annotateVariants(avaSet)
```

For an *AVASet* with corresponding annotated variants, the function `htmlReport` creates a html report containing variant and quality information.

The report is structured into three pages:

1. Variant report by reference: This page sums up additional information for each variant including name, type, reference gene, position, changed nucleotides and affected samples. In addition, every variant is linked to a page with further details about the affected genes and transcripts (e.g. Ensembl gene-IDs, transcript-IDs, codon sequences, changes of amino acids (if coding)).
2. Variant report by sample: The upper fraction of this page presents an overview of all samples together with links to individual amplicon coverage plots for each sample. In the lower fraction the found variants are listed for each sample separately in the same way as described in the variant report by reference above.
3. Quality report: The report shows the coverage of every amplicon in forward and/or reverse direction. Further plots display the coverage by MID and PTP (if this information is given in the pheno data of the object).

The following command creates a report containing only variants covered by at least 5% of the reads using the argument `minMut` (`minMut=3` is the default

value). The argument `blocks` can be used to structure the page by assigning each variant to a block. In this example the corresponding genes for each variant are used to create blocks, resulting in only one block in the example data set:

```
> blocks = as.character(sapply(annotatedVariants(avaAnnot),  
  function(x) x$genes$external_gene_id))  
> htmlReport(avaSet, annot=avaAnnot, blocks=blocks, dir="htmlReportExampleAVA",  
  title="htmlReport Example", minMut=3)
```

2.7 Plotting

2.7.1 Plot amplicon coverage

The function `plotAmpliconCoverage` creates a plot showing the coverage (number of reads) per amplicon, MID or PTP. This results in a barplot if the `AVASet` contains only one sample or in a boxplot for all other cases.

```
> plotAmpliconCoverage(avaSet[, 2], type="amplicon")
```

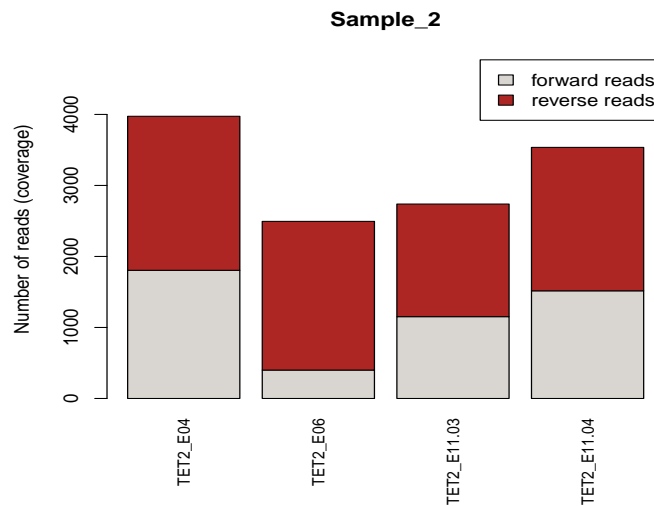


Figure 1: Barplot of the amplicon coverage for sample 2.

```
> plotAmpliconCoverage(avaSet, bothDirections=TRUE, type="amplicon")
```

2.7.2 Plot variation frequency

Given a Roche Amplicon Variant Analyzer Global Alignment export file, the function `plotVariationFrequency` creates a plot similar to the variation frequency plot in Roche's GS Amplicon Variant Analyzer. The plot shows the reference sequence along the x-axis and indicates variants as bars at the appropriate positions. The height of the bars corresponds to the percentage of reads carrying the variant. A second y-axis indicates the absolute number of reads covering the variant. `plotRange` defines the start and end base of the reference sequence that should be plotted.

```
> file = system.file("extdata", "AVAVarFreqExport", "AVAVarFreqExport.xls",
  package="R453Plus1Toolbox")
> plotVariationFrequency(file, plotRange=c(50, 150))
```

2.7.3 Plot variant locations

The function `plotVariants` illustrates the positions and types of mutations within a given `gene` and `transcript` (specified by an Ensembl gene/transcript id). The plot shows only coding regions (thus, units are amino acids / codons). The coding region is further divided into exons labeled with their rank in the transcript. An attribute `regions` allows to highlight special, predefined areas on the transcript like for example protein domains.

The function can be used in two ways:

It offers the most functionality when used as a "standalone" function by passing all mutations as a data frame. This mode allows an individual and detailed annotation of the mutations like labels, colors and user defined mutation types. It requires the columns "label", "pos" "mutation" and "color". It is recommended to add more detailed info for each mutation type by preparing a data frame for the parameter `mutationInfo` which requires the three columns "mutation", "legend" and "color".

The following example calls `plotVariants` for the gene TET2 having the Ensembl id "ENSG00000168769" and transcript "ENST00000513237" (see Figure 4 below):

```
> data(plotVariantsExample)
> geneInfo = plotVariants(data=variants, gene="ENSG00000168769",
  transcript="ENST00000513237", regions=regions,
  mutationInfo=mutationInfo, horiz=TRUE, cex=0.8)
```

Especially for integration into the R453Plus1Toolbox and for compatibility to older versions `plotVariants` also accepts annotated variants of class `annotatedVariants` (see section 2.6). The function then only distinguishes missense, nonsense and silent point mutations and deletions and does not include mutation labels.

3 Analysis of GS Mapper projects

Mapping projects allow the alignment of arbitrary reads from one or more sequencing runs to a given reference sequence.

3.1 Importing a GS Reference Mapper project

The function `MapperSet` imports data from Roche's GS Reference Mapper. The GS Mapper software stores information for each sample in a separate directory, so `MapperSet` expects a character vector `dirs` containing the directories of all samples to read in, i.e. directories containing the files:

- "mapping/454HCDiffs.txt"
- "mapping/454NewblerMetrics.txt"

Furthermore the parameter `samplenames` allows the separate specification of sample names. If missing, the directory names are taken. The following example imports a project containing 3 samples (N01, N03, N04) with a total of 111 variants:

```
> dir_sample01 = system.file("extdata", "MapperSet", "N01", package = "R453Plus1Toolbox")
> dir_sample03 = system.file("extdata", "MapperSet", "N03", package = "R453Plus1Toolbox")
> dir_sample04 = system.file("extdata", "MapperSet", "N04", package = "R453Plus1Toolbox")
> dirs = c(dir_sample01, dir_sample03, dir_sample04)
> mapperSet = MapperSet(dirs=dirs, samplenames=c("N01", "N03", "N04"))
```

3.2 The MapperSet class

An object of class `MapperSet` is a container to store data imported from a project of Roche's GS Reference Mapper Software. It directly extends the `Biobase eSet` class and as such provides the following slots:

1. The `assayData` slot contains four matrices with variants as rows and samples as columns:
 - `variantForwCount/variantRevCount`: Matrices containing the number of reads with the respective variant in forward/reverse direction.
 - `totalForwCount/totalRevCount`: Matrices containing the total read coverage for every variant location in forward/reverse direction.
2. The `featureData` slot holds the variants as rows together with additional information on each variant within the following columns:
 - `chromosome/start/end/strand`: Give the location of each variant.

- referenceBases/variantBase: Show the base(s) changed in each variant.
 - regName: The name of the region (gene) where the variant is located.
 - knownSNP: Contains dbSNP reference cluster ids for known SNPs as given by the GS Mapper software (if any).
3. The phenoData slot contains additional information about the samples represented as rows:
- By default, the phenoData slot only contains an accession number indicating the PTP of every sample.

```
> mapperSet
```

```
MapperSet (storageMode: list)
assayData: 111 features, 3 samples
  element names: variantForwCount, totalForwCount, variantRevCount, totalRevCount
protocolData: none
phenoData
  sampleNames: N01 N03 N04
  varLabels: accessionNumber
  varMetadata: labelDescription
featureData
  featureNames: 1 2 ... 111 (111 total)
  fvarLabels: chr strand ... knownSNP (8 total)
  fvarMetadata: labelDescription
experimentData: use 'experimentData(object)'
Annotation:
```

As the *MapperSet* is derived from the *Biobase eSet* the methods used to access or to manipulate the elements of a *MapperSet* object remain the same:

```
> assayData(mapperSet)$variantForwCount[1:4, ]
```

```

N01 N03 N04
1   9   7   7
2   9   6   6
3  11   3   5
4   7   5  11
```

```
> assayData(mapperSet)$totalForwCount[1:4, ]
```

```

N01 N03 N04
1   9   7   7
2   9   6   6
3  11   5   5
4   7   7  11
```

```

> fData(mapperSet)[1:4, ]

  chr strand  start      end referenceBases variantBase
1   1      + 11846252 11846252           G           A
2   1      + 11846447 11846447           G           A
3   1      + 11847340 11847340          ---          AGA
4   1      + 11847759 11847759           C           T
  regName  knownSNP
1  MTHFR  rs4846048
2  MTHFR  rs4845884
3  MTHFR  rs70983598
4  MTHFR  rs3737966

> pData(mapperSet)

  accessionNumber
N01      FZY3Q2K01
N03      FZY3Q2K01
N04      FZY3Q2K02

```

3.3 Setting filters and subsetting a MapperSet

The *MapperSet* uses the same methods for filtering and subsetting as the *AVASet* (see section 2.3 and 2.4 for details).

3.4 Annotations and Variant Reports

Before creating the variant and quality report, the variants have to be annotated using function `annotateVariants`. Annotation includes affected genes, exons and codons. Resulting amino acid changes are returned as well as dbSNP identifiers, if the mutation is already known. All information is fetched from Ensembl via *biomaRt* and returned in an object of class *AnnotatedVariants*. It is advisable to filter the *MapperSet* (see section 3.3) since the annotation process is very time consuming for a large number (>500) of variants.

```
> mapperAnnot = annotateVariants(mapperSet)
```

For a *MapperSet* with corresponding annotated variants, the function `htmlReport` creates a html report containing detailed variant information. The report is structured into two pages:

1. Variant report by reference: This page sums up additional information for each variant including name, type, reference gene, position, changed nucleotides and affected samples. Furthermore every variant is linked to a page with further details about the affected genes and transcripts (e.g. Ensembl gene-IDs, transcript-IDs, codon sequences, changes of amino acids (if coding)).

2. Variant report by sample: The upper fraction of this page presents an overview of all samples. In the lower fraction the found variants are listed for each sample separately in the same way as described in the variant report by reference above.

The following command creates a report containing only variants covered by at least 3% of the reads using the argument `minMut` (`minMut=3` is also the default value):

```
> htmlReport(mapperSet, annot=mapperAnnot, dir="htmlReportExampleMapper",
  title="htmlReport Example", minMut=3)
```

4 Detection of structural variants

Structural variants like translocations or inversions can be detected using non-paired reads if at least one read spans the breakpoint of the variant. These reads originate from two different locations on the reference genome and are called 'chimeric reads'.

4.1 Data preparation

Before breakpoints can be detected, the generated raw sequences must be pre-processed and aligned. Of course, data preprocessing depends on the applied laboratory protocols. The exemplary data set used in this vignette is a subset of the data set presented by Kohlmann et al. ([Kohlmann *et al.*, 2009]) and is described in detail therein.

In our example data set, each region of the pico titer plate contains reads from three different samples which were loaded into that region. To reallocate reads to samples, each sample has a unique multiplex sequence prefixing all reads from that sample. This allocation process is called *demultiplexing*. In the code section below, the multiplexed sequences are read in and demultiplexed according to the given multiplex sequences (MIDs) using the `demultiplexReads` method. The standard multiplex sequences used by the Genome Sequence MID library kits can be retrieved by calling `genomeSequencerMIDs`. The last two commands show that all reads could be successfully demultiplexed.

```
> fnaFile = system.file("extdata", "StructuralVariantDetection",
  "R_2009_07_30_14_26_52_FLX12080469_Administrator_714003",
  "D_2009_07_31_08_45_30_flxcluster_fullProcessing_714003",
  "1.TCA.454Reads.fna", package="R453Plus1Toolbox")
> seqs = readDNAStrngSet(fnaFile, format="fasta")
> MIDSeqs = genomeSequencerMIDs(c("MID1", "MID2", "MID3"))
> dmReads = demultiplexReads(seqs, MIDSeqs, numMismatches=2, trim=TRUE)
> length(seqs)
```



```
[1] 523
```

```
> sum(sapply(dmReads, length))
```

```
[1] 523
```

A sequence capture array was used to ensure that the example data set predominantly contains reads from certain genomic regions of interest. The applied NimbleGen array captured short segments corresponding to all exon regions of 92 distinct target genes. In addition, contiguous genomic regions for three additional genes, i.e. *CBFB*, *MLL*, and *RUNX1*, were present on the array. During sample preparation, linkers were ligated to the polished fragments in the library to provide a priming site as recommended by the NimbleGen protocol. These linker sequences were sequenced and are located at the 5 prime end of the reads. In case of long reads, the reverse complement of the linker may be located at the 3 prime end. The function `removeLinker` can be used to remove these linkers. Additionally, very short reads are discarded in the following code snippet.

```
> minReadLength = 15
> gSel3 = sequenceCaptureLinkers("gSel3")[[1]]
> trimReads = lapply(dmReads, function (reads) {
  reads = reads[width(reads) >= minReadLength]
  reads = removeLinker(reads, gSel3)
  reads = reads[width(reads) >= minReadLength]
  readsRev = reverseComplement(reads)
  readsRev = removeLinker(readsRev, gSel3)
  reads = reverseComplement(readsRev)
  reads = reads[width(reads) >= minReadLength]
  return(reads)
})
```

Finally, the preprocessed reads must be aligned against a reference genome. For this purpose, we write the reads to a .fasta file and use the BWA-SW ([Li and Durbin, 2010]) algorithm for generating local alignments. The BWA-SW algorithm can be substituted by other local alignment algorithms. However, BWA-SW has the useful feature to only report the best local alignments. Hence, two local alignments do not overlap on the query sequence (they may overlap on the reference). This is an assumption made by the pipeline implemented in this package.

```
> write.XStringSet(trimReads[["MID1"]], file="/tmp/NO1.fasta", format="fasta")
```

4.2 Computing and assessing putative structural variants

As chimeric reads may also be caused by technical issues during sample preparation, the function `filterChimericReads` implements several filter steps to

remove artificial chimeric reads.

The remaining reads are passed to the `detectBreakpoints` method to create clusters representing putative breakpoints. Each cluster contains all chimeric reads that span this breakpoint. Promising candidates are clusters with more than one read and ideally with reads from different strands. Some structural variations like translocations or inversion lead to two related breakpoints. In the context of fusion genes, these breakpoints are referred to as *pathogenic* and *reciprocal* breakpoint. By the use of read orientation and strand information during clustering, it is ensured that reads from the pathogenic breakpoint will not cluster together with reads from the reciprocal breakpoint, although their genomic coordinates may be close to each other or even equal. After clustering, consensus breakpoint coordinates are computed for each cluster.

In the last step, the function `mergeBreakpoints` searches breakpoints that originate from the same structural variation (i.e. the pathogenic and the related reciprocal breakpoint) and merges them. We observed, that the distance between two related breakpoints may be up to a few hundred basepairs, whereas the breakpoint coordinates of single reads spanning the same breakpoint vary only by a very few bases due to sequencing errors or ambiguities during alignment.

In the following example, we use the reads from sample N01 presented in the previous section. The reads have been aligned using BWA-SW:

```
> library("Rsamtools")
> bamFile = system.file("extdata", "StructuralVariantDetection", "bam", "N01.bam",
  package="R453Plus1Toolbox")
> parameters = ScanBamParam(what=scanBamWhat())
> bam = scanBam(bamFile, param=parameters)
```

For the filtering step, we specify a target region, i.e. the used capture array in form of a *RangesList*. All chimeric reads not overlapping this region with at least one local alignment are discarded. The following example creates a target region out of a given `.bed` file containing region information using functions from package *rtracklayer*.

```
> library("rtracklayer")
> bedFile = system.file("extdata", "StructuralVariantDetection", "chip",
  "CaptureArray_hg19.bed", package="R453Plus1Toolbox")
> chip = import.ucsc(bedFile, subformat="bed")
> chip = ranges(chip[[1]])
> names(chip) = gsub("chr", "", names(chip))
> linker = sequenceCaptureLinkers("gSe13")[[1]]
> filterReads = filterChimericReads(bam, targetRegion=chip, linkerSeq=linker)
> filterReads$log
```

	AlignedReads	ChimericReads	TwoLocalAlignments	
1	213	24	24	
	TargetRegion	NoLinker	MinimumDistance	Unique5PrimeStart
1	23	23	23	23

The `linkerSeq` argument allows to specify the linker sequence used during sample preparation. All chimeric reads that have this linker sequence between their local alignments are removed.

Finally, we call the `detectBreakpoints` and `mergeBreakpoints` functions:

```
> bp = detectBreakpoints(filterReads, minClusterSize=1)
> bp
```

	Size	ChrA	ChrB
BP1	8	16	16
BP2	4	16	16
BP3	1	21	1
BP4	1	2	1
BP5	1	1	7
BP6	1	1	16

```
> table(bp)
```

```
size
 1  4  8
11  1  1
```

```
> mbp = mergeBreakpoints(bp)
> summary(mbp)
```

	ChrA	ChrB	BpACase1	BpBCase1	BpACase2	BpBCase2
BP1_BP2	16	16	15815191	67121088	15815189	67121086
BP3	21	1	36496155	177984464	NA	NA
BP4	2	1	16382474	186276897	NA	NA
BP5	1	7	186275614	102017970	NA	NA
BP6	1	16	186271118	67130495	NA	NA
BP7	1	11	174926056	118389220	NA	NA
BP8	1	16	120222145	15853548	NA	NA
BP9	6	1	168290170	178598476	NA	NA
BP10	21	1	37093848	150600467	NA	NA
BP11	15	1	63213753	164769097	NA	NA
BP12	21	1	36450706	186733804	NA	NA
BP13	1	21	192053733	37167301	NA	NA
	NoReadsCase1	NoReadsCase2	NoReadsTotal			
BP1_BP2	4/4	3/1	12			
BP3	1/0	0/0	1			

BP4	1/0	0/0	1
BP5	1/0	0/0	1
BP6	1/0	0/0	1
BP7	1/0	0/0	1
BP8	1/0	0/0	1
BP9	1/0	0/0	1
BP10	1/0	0/0	1
BP11	1/0	0/0	1
BP12	1/0	0/0	1
BP13	1/0	0/0	1

One cluster of size 8 and another cluster of size 4 were detected. Both putative breakpoints span two regions on chromosome 16. Further, 11 clusters of size one were found. The `mergeBreakpoints` function merges the first two clusters. The summary reveals that the coordinates of the breakpoints only differ by two bases at each region on chromosome 16. Moreover, both strands from both breakpoints were sequenced. Obviously, we detected two related breakpoints caused by an inversion on chromosome 16.

4.3 Visualization of breakpoints

The function `plotChimericReads` takes the output of the function `mergeBreakpoints` and produces a plot of the breakpoint regions together with the aligned reads and marks deletions, insertions and mismatches. If a pathogenic and a reciprocal breakpoint exist, `plotChimericReads` creates two plots as shown in the example below.

The following example shows the breakpoints (pathogenic and reciprocal) of an inversion on chromosome 16 where 12 reads aligned:

```
> plotChimericReads(mbp[1], legend=TRUE)
```

Optionally (if the argument `plotBasePairs` is `TRUE`), `plotChimericReads` displays all base pairs within a given region of size `maxBasePairs` around the breakpoint:

```
> plotChimericReads(mbp[1], plotBasePairs=TRUE, maxBasePairs=30)
```

References

[Kohlmann *et al.*, 2009] Kohlmann, A. *et al.* (2009) Targeted next-generation sequencing (NGS) enables for the first time the detection of point mutations, molecular insertions and deletions, as well as leukemia-specific fusion genes in AML in a single procedure. *Blood (ASH Annual Meeting Abstracts)*, **114**(22), 294–295.

[Li and Durbin, 2010] Li,H. and Durbin,R. (2010) Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*, **26**(5), 589–95.

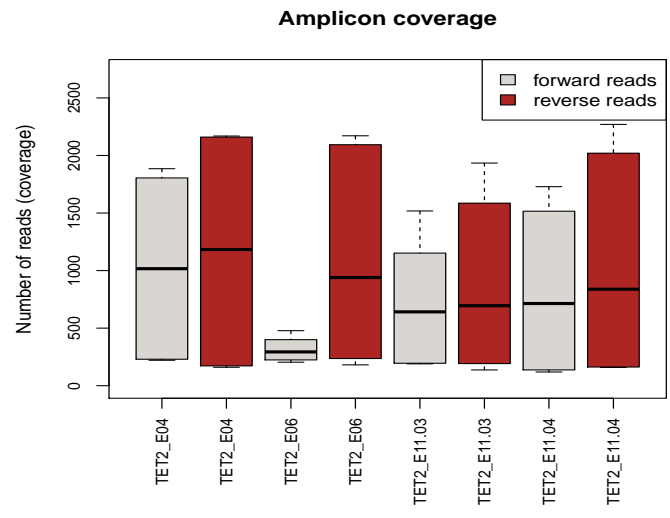


Figure 2: Boxplot of the coverage for four amplicons separated by read direction.

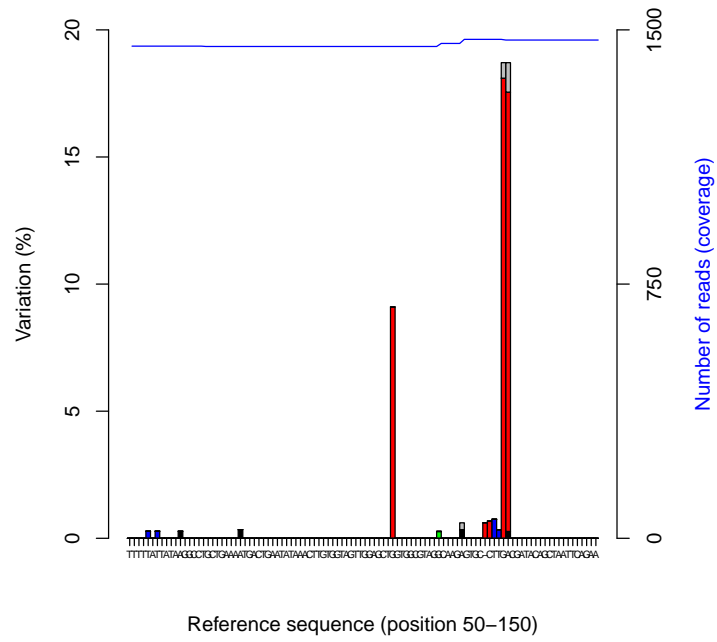


Figure 3: Plot of the variation frequency for a given reference sequence.

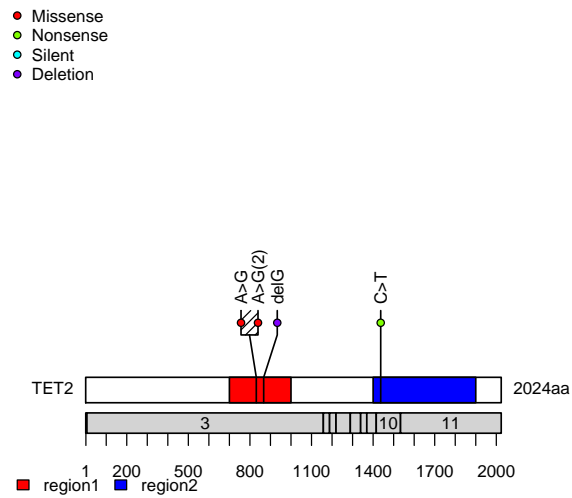


Figure 4: Plot of the variants for gene TET2 by passing mutations as a data frame. This version includes mutation labels and allows user defined mutation types.

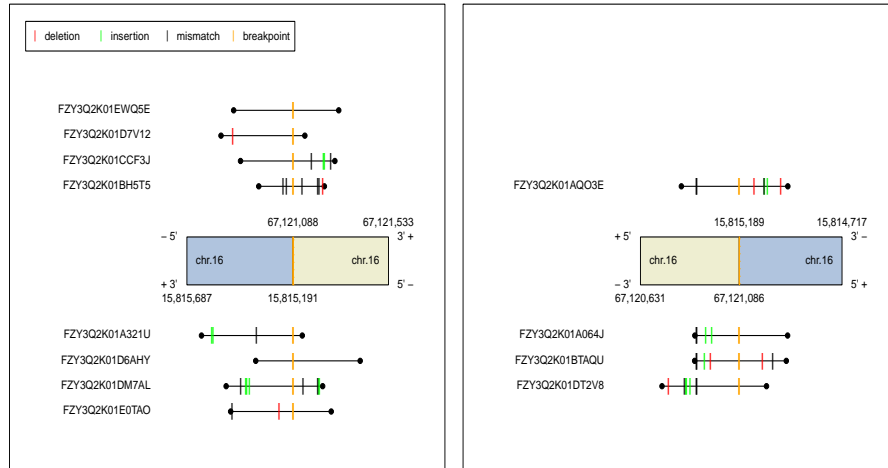


Figure 5: Plot of the breakpoint region.

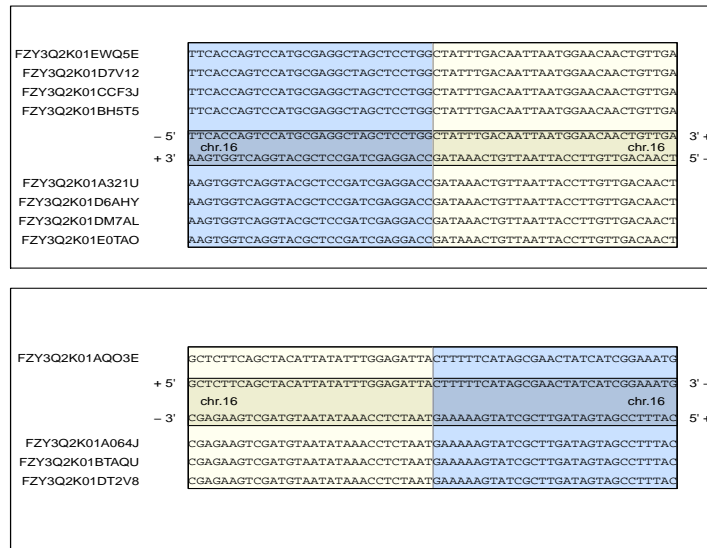


Figure 6: Plot of the breakpoint region including base pairs.