crispRvariants User Guide

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Introduction

The CRISPR-Cas9 system is an efficient method of introducing mutations into genomic DNA. A guide RNA directs nuclease activity to a 20 nucleotide target region, resulting in efficient mutagenesis. Repair of the cleaved DNA can introduce insertions and deletions centred around the cleavage site. Once the target sequence is mutated, the guide RNA will no longer bind and the DNA will not be cleaved again. SNPs within the target region, depending on their location, may also disrupt cleavage. The efficiency of a CRISPR-Cas9 experiment is typically measured by amplifying and sequencing the region surrounding the target sequence, then counting the number of sequenced reads that have insertions and deletions at the target site. The **crispRvariants** package formalizes this process and takes care of various details of managing and manipulating data for such confirmatory and exploratory experiments.

This guide shows an example illustrating how raw data is preprocessed and mapped and how mutation information is extracted relative to the reference sequence. The package comprehensively summarizes and plots the spectrum of variants introduced by CRISPR-Cas9 or similar genome editing experiments.

Case study 1: Analysis of ptena mutant spectrum in zebrafish (data from Mosimann laboratory, UZH)

Convert AB1-format Sanger sequences to FASTQ

This data set is from 5 separate clutches of fish (1 control - uninjected, 2 injected with strong phenotype, 2 injected with mild phenotype), with injections from a guide against the *ptena* gene. For this exercise, the raw data comes as AB1 (Sanger) format. To convert AB1 files to FASTQ, we use ab1ToFastq(), which is a wrapper for functions in **sangerseqR** package with additional quality score trimming.

Although there are many ways to organize such a project, we organize the data (raw and processed) data into a set of directories, with a directory for each type of data (e.g., ‘ab1’ for AB1 files, ‘fastq’ for FASTQ files, ‘bam’ for BAM files, etc.); this can continue with directories for scripts, for figures, and so on. With this structure in place, the following code sets up various directories.

```r
library(crispRvariants)
library(sangerseqR)

# List AB1 filenames, get sequence names,  make names for the fastq files
data_dir <- "ab1/ptena"
fq_dir <- "fastq"
ab1_fnames <- dir(data_dir, "ab1$", recursive=TRUE, full=TRUE)
sq_nms <- gsub(".ab1","",basename(ab1_fnames))

# Replace spaces and slashes in filename with underscores
fq_fnames  <- paste0(gsub("[\ |\\/]", "_", dirname(ab1_fnames)), ".fastq")

# abifToFastq to read AB1 files and write to FASTQ
dummy <- mapply( function(u,v,w) {
        abifToFastq(u,v,file.path(fq_dir,w))
}, sq_nms, ab1_fnames, fq_fnames)
```

We will collect sequences from each embryo into the same FASTQ file. Note that abifToFastq *appends* output to existing files where possible. In this experiment, there are 40 sequences, which will be output to 5 files:

```
length(unique(ab1_fnames))
```

```
## [1] 40
```

```
length(unique(fq_fnames))
```

```
## [1] 5
```

Some of the AB1 files may not have a sufficient number of bases after quality score trimming (default is 20 bases). In these cases, abifToFastq() issues a warning (suppressed here).

Map the fastq reads

We use FASTQ format because it is the major format used by most genome alignment algorithms. At this stage, the alignment *could* be done outside of R (e.g., using command line tools), but below we use R and a call to system() to keep the whole workflow within R. Note that this also requires various software tools (e.g., **bwa**, **samtools**) to already be installed.

The code below iterates through all the FASTQ files generated above and aligns them to a pre-indexed genome.

```
library("Rsamtools")

# BWA indices were generated using bwa version 0.7.10
bwa_index <- "GRCHz10.fa.gz"
bam_dir <- "bam"
fq_fnames <- file.path(fq_dir,unique(fq_fnames))
bm_fnames <- gsub(".fastq$",".bam",basename(fq_fnames))
srt_bm_fnames <- file.path(bam_dir, gsub(".bam","_s",bm_fnames))

# Map, sort and index the bam files, remove the unsorted bams
for(i in 1:length(fq_fnames)) {
  cmd <- paste0("bwa mem ", bwa_index, " ", fq_fnames[i],
                " | samtools view -Sb - > ", bm_fnames[i])
  message(cmd, "\n"); system(cmd)
  indexBam(sortBam(bm_fnames[i],srt_bm_fnames[i]))
  unlink(bm_fnames[i])
}
```

See the help for **bwa index** at the bwa man page and for general details on mapping sequences to a genome reference.

Read in bam files and initialize CrisprSet object

Given a set of BAM files with the amplicon sequences of interest mapped to the reference genome, we need to collect a few additional pieces of information about the guide sequence and define the area around the guide that we want to summarize the mutation spectrum over.

If you already know the coordinates, these can be typed in or put in a BED file that can be read in using the **rtracklayer** package. The import() below command returns a GRanges object.

```r
library(rtracklayer)
# Represent the guide as a GenomicRanges::GRanges object
gd <- import("bed/guide.bed")
gd
```

```
## GRanges object with 1 range and 2 metadata columns:
##       seqnames               ranges strand |        name     score
##          <Rle>            <IRanges>  <Rle> | <character> <numeric>
##   [1]    chr17 [23648474, 23648496]      - |   ptena_ccA         0
##   -------
##   seqinfo: 1 sequence from an unspecified genome; no seqlengths
```

Below, we'll extend the guide region by 5 bases on each side when counting variants. The guide designed for *ptena* (including PAM) is 23bp and is located on chromosome chr17 from 23648474-23648496. Note that the expected cut site (used later for labeling variants), after extension, is at base 22 with respect to the start of the guide sequence.

```r
gdl <- resize(gd, width(gd) + 10, fix = "center")
```

With the Bioconductor **BSgenome** packages, the reference sequence itself can be retrieved directly into a DNAStringSet object. For other genomes, the reference sequence can be retrieved from a genome by first indexing the genome with samtools faidx and then fetching the required region. Here we are using the GRCHz10 zebrafish genome. The reference sequence was fetched and saved as follows:

```r
system("samtools faidx GRCHz10.fa.gz")

reference=system(sprintf("samtools faidx GRCHz10.fa.gz %s:%s-%s",
                         seqnames(gdl)[1], start(gdl)[1], end(gdl)[1]) ,
                 intern = TRUE)[[2]]

# The guide is on the negative strand, so the reference needs to be reverse complemented
reference=reverseComplement(DNAString(reference))
save(reference, file = "ptena_GRCHz10_ref.rda")
```

We'll load the previously saved reference sequence.

```r
load("ptena_GRCHz10_ref.rda")
reference
```

```
##   33-letter "DNAString" instance
## seq: GCCATGGGCTTTCCAGCCGAACGATTGGAAGGT
```

Note the NGG sequence (here, TGG) is present with the 5 extra bases on the end.

To allow easy matching to experimental condition (e.g., useful for colour labeling) and for subsetting to experiments of interest, we often organize the list of BAM files together with accompanying metadata in a machine-readable table beforehand:

```
# The metadata and bam files for this experiment are included with crisprRvariants
library("gdata")
md_fname <- "metadata/metadata.xls"
md <- read.xls(md_fname, 1)
md
```

```
##                                     bamfile                       directory
## 1        ab1_ptena_phenotype_embryo_1_s.bam        ptena/phenotype/embryo 1
## 2        ab1_ptena_phenotype_embryo_2_s.bam        ptena/phenotype/embryo 2
## 3 ab1_ptena_wildtype_looking_embryo_1_s.bam ptena/wildtype looking/embryo 1
## 4 ab1_ptena_wildtype_looking_embryo_2_s.bam ptena/wildtype looking/embryo 2
## 5       ab1_ptena_uninjected_embryo_1_s.bam       ptena/uninjected/embryo 1
##   Short.name Targeting.type    sgRNA1 sgRNA2   Group
## 1    ptena 1         single ptena_ccA     NA  strong
## 2    ptena 2         single ptena_ccA     NA  strong
## 3    ptena 3         single ptena_ccA     NA    mild
## 4    ptena 4         single ptena_ccA     NA    mild
## 5    control         single ptena_ccA     NA control
```

```
# Get the bam filenames from the metadata table
bam_dir <- "bam"
bam_fnames <- file.path(bam_dir, md$bamfile)

# check that all files exist
all( file.exists(bam_fnames) )
```

```
## [1] TRUE
```

Creating a CrisprSet

The next step is to create a CrisprSet object, which is the container that stores the relevant sequence information, alignments, observed variants and their frequencies.

```
# Note that the zero point (target.loc parameter) is 22
crispr_set <- readsToTarget(bam_fnames, target = gdl, reference = reference,
                            names = md$Short.name, target.loc = 22)
```

```
## [1] "Initialising CrisprSet chr17:23648469-23648501 with 5 samples"
```

```
crispr_set
```

```
## CrisprSet object containing 5 CrisprRun samples
## Target location:
## GRanges object with 1 range and 2 metadata columns:
##       seqnames               ranges strand |        name     score
##          <Rle>            <IRanges>  <Rle> | <character> <numeric>
##   [1]    chr17 [23648469, 23648501]      - |   ptena_ccA         0
##   -------
##   seqinfo: 1 sequence from an unspecified genome; no seqlengths
## [1] "Most frequent variants:"
##            ptena 1 ptena 2 ptena 3 ptena 4 control
## no variant       3       4       4       0       7
## -1:4D            0       0       0       2       0
## 6:1D             0       0       0       1       1
## 1:7I             1       0       0       0       0
## 2:1D,4:5I        0       0       0       1       0
## Other            0       0       1       1       0
```

```
# The counts table can be accessed with the "variantCounts" function
vc <- variantCounts(crispr_set)
print(class(vc))
```

```
## [1] "matrix"
```

You can see that in the table of variant counts, variants are summarised by the location of their insertions and deletions with respect to the target site. Non-variant sequences and sequences with a single nucleotide variant (SNV) but no insertion or deletion (indel) are displayed first, followed by the indel variants from most to least frequent For example, the most frequent non-wild-type variant, "-1:4D" is a 4 base pair deletion starting 1 base upstream of the zero point.

Creating summary plots of variants

We want to plot the variant frequencies along with the location of the guide sequence relative to the known transcripts. If you do this repeatedly for the same organism, it is worthwhile to save the database in a local file and read in with loadDb(), since this is quicker than retrieving it from UCSC (or Ensembl) each time.

We start by creating a transcript database of Ensembl genes. The gtf was downloaded from Ensembl version 81. We first took a subset of just the genes on chromosome 17 and then generated a transcript database.

```
# Extract genes on chromosome 17 (command line)
# Note that the Ensembl gtf does not include the "chr" prefix, so we add it here
gtf=Danio_rerio.GRCz10.81.gtf.gz
zcat ${gtf} | awk '($1 == 17){print "chr"$0}' > Danio_rerio.GRCz10.81_chr17.gtf
```

```
## gzip: Danio_rerio.GRCz10.81.gtf.gz: No such file or directory
```

```
# In R
library(GenomicFeatures)
txdb <- makeTxDbFromGFF("Danio_rerio.GRCz10.81_chr17.gtf", format = "gtf")
saveDb(txdb, file= "GRCz10_81_chr17_txdb.sqlite")
```

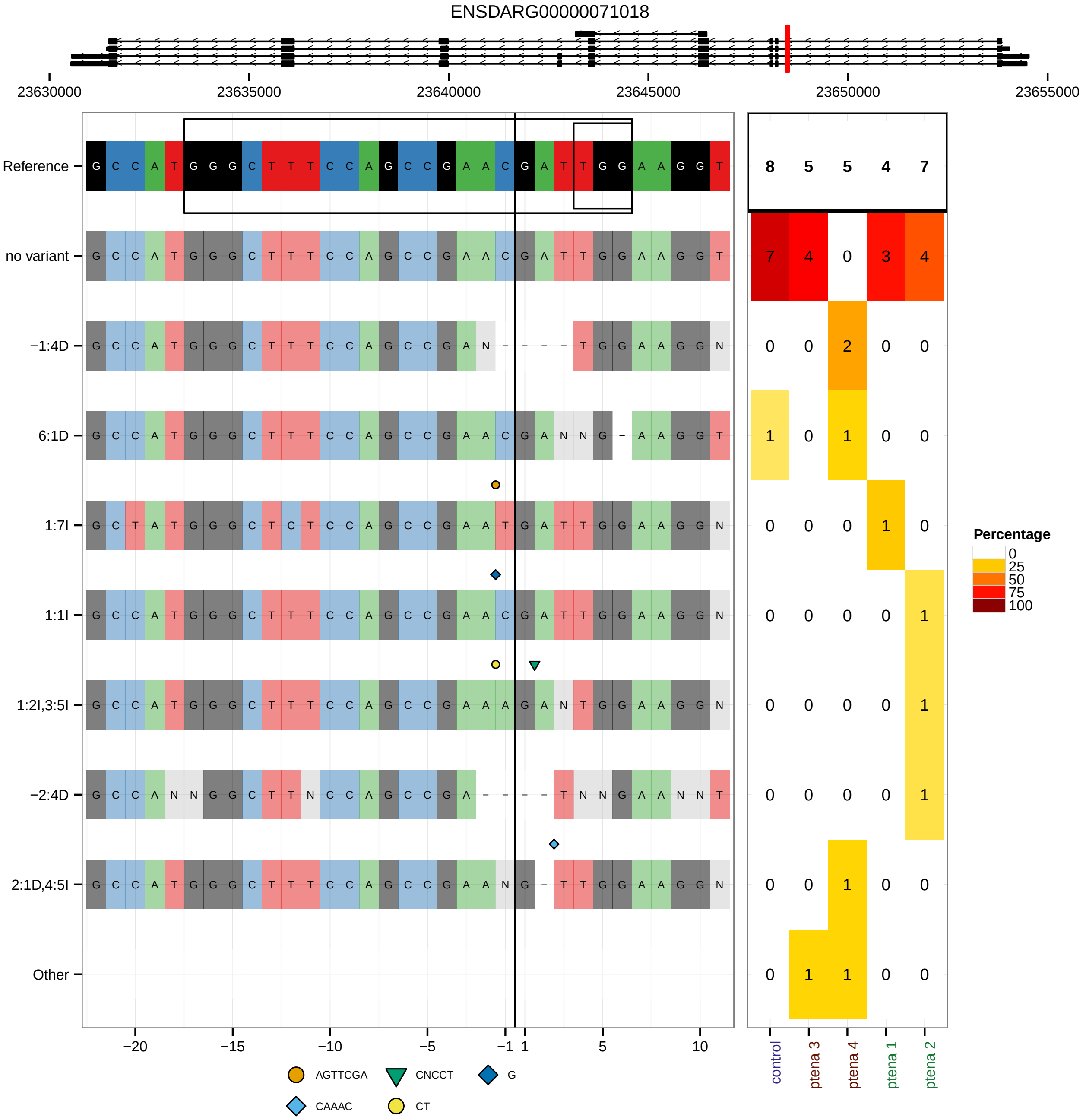
We now load the the previously saved database

plotVariants() is a wrapper function that groups together a plot of the transcripts of the gene/s overlapping the guide (optional), crispRvariants::plotAlignments(), which displays the alignments of the consensus variant sequences to the reference, and crispRvariants::plotFreqHeatmap(), which produces a table of the variant counts per sample, coloured by either their counts or percentage contribution to the variants observed for a given sample. If a transcript database is supplied, the transcript plot is annotated with the guide location. Arguments for plotAlignments() and plotFreqHeatmap() can be passed to plotVariants() as lists named plotAlignments.args and plotFreqHeatmap.args, respectively.

```r
# The gridExtra package is required to specify the legend.key.height
# as a "unit" object.  It is not needed to call plotVariants() with defaults
library(gridExtra)

# Match the clutch id to the column names of the variants
group <- md$Group
```

```r
p <- plotVariants(crispr_set, txdb = txdb, gene.text.size = 8,
    row.ht.ratio = c(1,8), col.wdth.ratio = c(4,2),
    plotAlignments.args = list(line.weight = 0.5, ins.size = 2,
                               legend.symbol.size = 4),
    plotFreqHeatmap.args = list(plot.text.size = 3, x.size = 8, group = group,
                                legend.text.size = 8,
                                legend.key.height = grid::unit(0.5, "lines")))
```



The plotVariants() options set the text size of the transcript plot annotation (gene.text.size) and the relative heights (row.ht.ratio) and widths (col.wdth.ratio) of the plots.

The plotAlignments arguments set the symbol size in the figure (ins.size) and in the legend (legend.symbol), the line thickness for the (optional) annotation of the guide region and cleavage site (line.weight).

For plotFreqHeatmap we define an grouping variable for colouring the x-axis labels (group), the size of the text within the plot (plot.text.size) and on the x-axis (x.size) and set the size of the legend text (legend.text.size).

Calculating the mutation efficiency

The mutation efficiency is the number of reads that include an insertion or deletion. Chimeric reads and reads containing single nucleotide variants near the cut site may be counted as variant reads, non-variant reads, or excluded entirely. See the help page for the function **mutationEfficiency** for more details.

We can see in the plot above that the control sample includes a variant sequence 6:1D, also present in sample ptena 4. We will exclude all sequences with this variant from the efficiency calculation. We also demonstrate below how to exclude particular variants.

```
# Calculate the mutation efficiency, excluding indels that occur in the "control" sample
# and further excluding the "control" sample from the efficiency calculation
eff <- mutationEfficiency(crispr_set, filter.cols = "control", exclude.cols = "control")
eff
```

```
##   ptena 1   ptena 2   ptena 3   ptena 4   Average    Median   Overall ReadCount
##     25.00     42.86     20.00     80.00     41.96     33.93     42.86     21.00
```

```
# Suppose we just wanted to filter particular variants, not an entire sample.
# This can be done using the "filter.vars" argument
eff2 <- mutationEfficiency(crispr_set, filter.vars = "6:1D", exclude.cols = "control")

# The results are the same in this case as only one variant was filtered from the control
identical(eff,eff2)
```

```
## [1] TRUE
```

We see above that sample ptena 4 has an efficiency of 80%, i.e. 4 variant sequences, plus one sequence “6:1D” which is counted as a non-variant sequence as it also occurs in the control sample.

Plot chimeric alignments

When deciding whether chimeric alignments should be considered as variant sequences, it can be useful to plot the frequent chimeras.

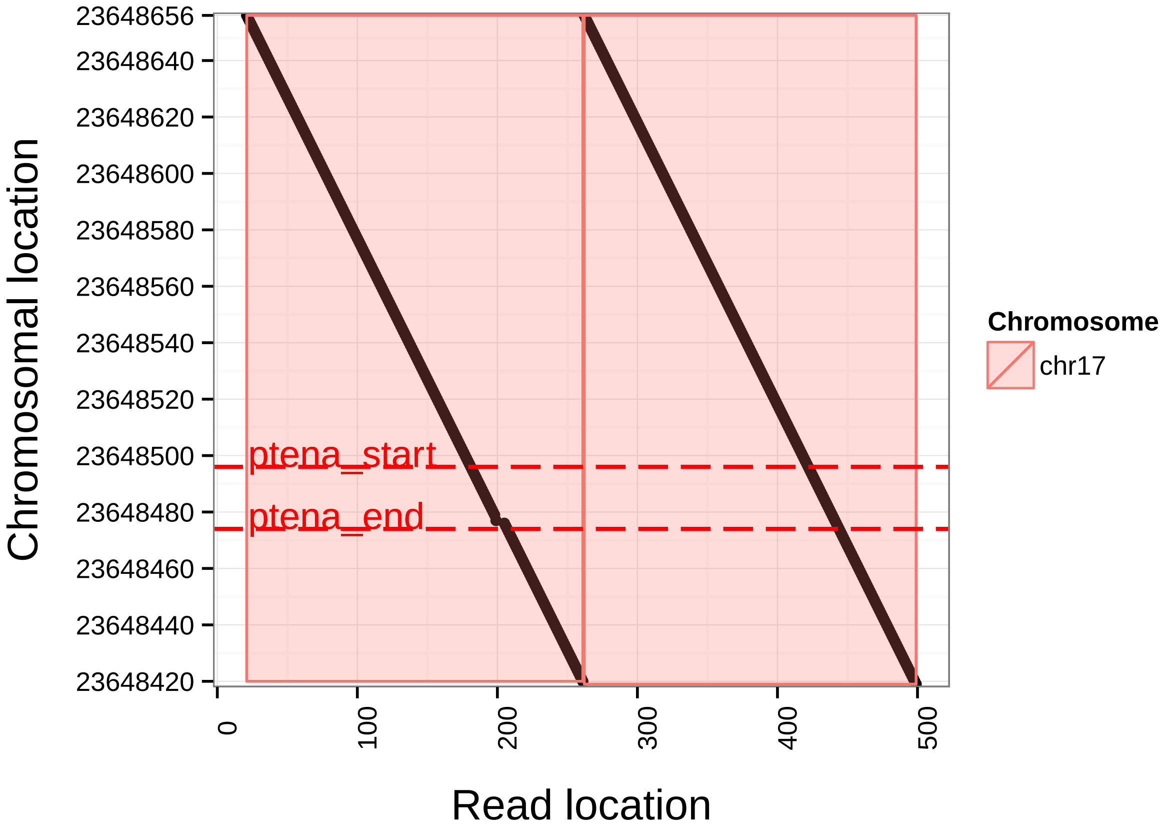
```
ch <- getChimeras(crispr_set, sample = "ptena 4")

# Confirm that all chimeric alignments are part of the same read
length(unique(names(ch))) == 1
```

```
## [1] TRUE
```

```
# Set up points to annotate on the plot
annotations <- c(resize(gd, 1, fix = "start"), resize(gd, 1, fix = "end"))
annotations$name <- c("ptena_start", "ptena_end")

plotChimeras(ch, annotations = annotations)
```



Here we see the read aligns as two tandem copies of the region chr17:23648420-23648656. The endpoint of each copy is not near the guide sequence. We do not consider this a genuine mutation, so we'll recalculate the mutation efficiency excluding the chimeric reads and the control variant as before.

```
mutationEfficiency(crispr_set, filter.cols = "control", exclude.cols = "control",
                   include.chimeras = FALSE)
```

```
##   ptena 1   ptena 2   ptena 3   ptena 4   Average    Median   Overall ReadCount
##     25.00     42.86      0.00     75.00     35.71     33.93     36.84     19.00
```

We see that the mutation effiency for "ptena 4" is now 75%, i.e. 3 genuine variant sequences, 1 sequence counted as "non-variant" because it occurs in the control, and the chimeric read excluded completely.