BASELINe quantifies selection pressure by calculating the posterior probability density function (PDF) based on observed mutations compared to expected mutation rates derived from an underlying SHM targeting model. Selection is quantified via the following steps:

1. Calculate the selection scores for individual sequences.
2. Group by relevant fields for comparison and convolve individual selection PDFs.
3. Plot and compare selection scores of different groups of sequences.

Example data

A small example AIRR Rearrangement database is included in the alakazam package. The example dataset consists of a subset of Ig sequencing data from an influenza vaccination study (Laserson and Vigneault et al., PNAS, 2014). The data include sequences from multiple time-points before and after the subject received an influenza vaccination. Quantifying selection requires the following fields (columns) to be present in the table:

- sequence_id
- sequence_alignment
- germline_alignment_d_mask

```r
# Load example data
library(shazam)
data(ExampleDb, package="alakazam")
```

Calculate selection PDFs for individual sequences

Selection scores are calculated with the calcBaseline function. This can be performed with a single call to calcBaseline, which performs all required steps. Alternatively, one can perform each step separately for greater control over the analysis parameters.
Constructing clonal consensus sequences

Individual sequences within clonal groups are not, strictly speaking, independent events and it is generally appropriate to only analyze selection pressures on an effective sequence for each clonal group. The `collapseClones` function provides one strategy for generating an effective sequences for each clone. It reduces the input database to one row per clone and appends `clonal_sequence` and `clonal_germline` columns which contain the consensus sequences for each clone.

```r
# Collapse clonal groups into single sequences
clones <- collapseClones(ExampleDb, cloneColumn="clone_id",
                        sequenceColumn="sequence_alignment",
                        germlineColumn="germline_alignment_d_mask",
                        regionDefinition=IMGT_V,
                        method="thresholdedFreq", minimumFrequency=0.6,
                        includeAmbiguous=FALSE, breakTiesStochastic=FALSE,
                        nproc=1)
```

Calculating selection in multiple steps

Following construction of an effective sequence for each clone, the observed and expected mutation counts are calculated for each sequence in the `clonal_sequence` column relative to the `clonal_germline`. `observedMutations` is used to calculate the number of observed mutations and `expectedMutations` calculates the expected frequency of mutations. The underlying targeting model for calculating expectations can be specified using the `targetingModel` parameter. In the example below, the default `HH_S5F` is used. Column names for sequence and germline sequence may also be passed in as parameters if they differ from the Change-O defaults.

Mutations are counted by these functions separately for complementarity determining (CDR) and framework (FWR) regions. The `regionDefinition` argument defines whether these regions are handled separately, and where the boundaries lie. There are two built-in region definitions in the `shazam` package, both dependent upon the V segment being IMGT-gapped:

- **IMGT_V**: All regions in the V segment, excluding CDR3, grouped as either CDR or FWR.
- **IMGT_V_BY_REGIONS**: The CDR1, CDR2, CDR3, FWR1, FWR and FWR3 regions in the V segment (no CDR3) treated as individual regions.

Users may define other region sets and boundaries by creating a custom `RegionDefinition` object.

```r
# Count observed mutations and append mu_count columns to the output
observed <- observedMutations(clones,
                               sequenceColumn="clonal_sequence",
                               germlineColumn="clonal_germline",
                               regionDefinition=IMGT_V, nproc=1)

# Count expected mutations and append mu_expected columns to the output
expected <- expectedMutations(observed,
                               sequenceColumn="clonal_sequence",
                               germlineColumn="clonal_germline",
                               targetingModel=HH_S5F,
                               regionDefinition=IMGT_V, nproc=1)
```

The counts of observed and expected mutations can be combined to test for selection using
calcBaseline. The statistical framework used to test for selection based on mutation counts can be specified using the testStatistic parameter.

# Calculate selection scores using the output from expectedMutations
baseline <- calcBaseline(expected, testStatistic="focused",
                        regionDefinition=IMGT_V, nproc=1)

Calculating selection in one step
It is not required for observedMutation and expectedMutations to be run prior to calcBaseline. If the output of these two steps does not appear in the input data.frame, then calcBaseline will call the appropriate functions prior to calculating selection scores.

# Calculate selection scores from scratch
baseline <- calcBaseline(clones, testStatistic="focused",
                        regionDefinition=IMGT_V, nproc=1)

Using alternative mutation definitions and models
The default behavior of observedMutations and expectedMutations, and by extension calcBaseline, is to define a replacement mutation in the usual way - any change in the amino acid of a codon is considered a replacement mutation. However, these functions have a mutationDefinition argument which allows these definitions to be changed by providing a MutationDefinition object that contains alternative replacement and silent criteria. shazam provides the following built-in MutationDefinitions:

- CHARGE_MUTATIONS: Amino acid mutations are defined by changes in side chain charge class.
- HYDROPATHY_MUTATIONS: Amino acid mutations are defined by changes in side chain hydrophobicity class.
- POLARITY_MUTATIONS: Amino acid mutations are defined by changes in side chain polarity class.
- VOLUME_MUTATIONS: Amino acid mutations are defined by changes in side chain volume class.

The default behavior of expectedMutations is to use the human 5-mer mutation model, HH_S5F. Alternative SHM targeting models can be provided using the targetingModel argument.

# Calculate selection on charge class with the mouse 5-mer model
baseline <- calcBaseline(clones, testStatistic="focused",
                        regionDefinition=IMGT_V,
                        targetingModel=MK_RS5NF,
                        mutationDefinition=CHARGE_MUTATIONS,
                        nproc=1)

Group and convolve individual selection distributions
To compare the selection scores of groups of sequences, the sequences must be convolved into a single PDF representing each group. In the example dataset, the sample_id field corresponds to samples taken at different time points before and after an influenza vaccination and the c_call field specifies the isotype of the sequence. The groupBaseline function convolves the BASELINe PDFs of individual sequences/clones to get a combined PDF. The field(s) by which to group the sequences are specified with the groupBy parameter.
The `groupBaseline` function automatically calls `summarizeBaseline` to generate summary statistics based on the requested groupings, and populates the `stats` slot of the input `Baseline` object with the number of sequences with observed mutations for each region, mean selection scores, 95% confidence intervals, and p-values with positive signs indicating the presence of positive selection and/or p-values with negative signs indicating the presence of negative selection. The magnitudes of the p-values (without the signs) should be interpreted as analogous to a t-test.

**Grouping by a single annotation**

The following example generates a single selection PDF for each unique annotation in the `sample_id` column.

```r
# Combine selection scores by time-point
grouped_1 <- groupBaseline(baseline, groupBy="sample_id")
```

**Subsetting and grouping by multiple annotations**

Grouping by multiple annotations follows the sample procedure as a single annotation by simply adding columns to the `groupBy` argument. Subsetting the data can be performed before or after generating selection PDFs via `calcBaseline`. However, note that subsetting may impact the clonal representative sequences generated by `collapseClones`. In the following example subsetting precedes the collapsing of clonal groups.

```r
# Subset the original data to switched isotypes
db_sub <- subset(ExampleDb, c_call %in% c("IGHM", "IGHG"))

# Collapse clonal groups into single sequence
clones_sub <- collapseClones(db_sub, cloneColumn="clone_id",
sequenceColumn="sequence_alignment",
germlineColumn="germline_alignment_d_mask",
regionDefinition=IMGT_V,
method="thresholdedFreq", minimumFrequency=0.6,
includeAmbiguous=FALSE, breakTiesStochastic=FALSE,
nproc=1)

# Calculate selection scores from scratch
baseline_sub <- calcBaseline(clones_sub, testStatistic="focused",
regionDefinition=IMGT_V, nproc=1)

# Combine selection scores by time-point and isotype
grouped_2 <- groupBaseline(baseline_sub, groupBy=c("sample_id", "c_call"))
```

**Convolving variables at multiple levels**

To make selection comparisons using two levels of variables, you would need two iterations of groupings, where the first iteration of `groupBaseline` groups on both variables, and the second iteration groups on the “outer” variable. For example, if a data set has both case and control subjects, annotated in `status` and `subject` columns, then generating convolved PDFs for each status would be performed as:
# First group by subject and status
subject_grouped <- groupBaseline(baseline, groupBy=c("status", "subject"))

# Then group the output by status
status_grouped <- groupBaseline(subject_grouped, groupBy="status")

Testing the difference in selection PDFs between groups

The testBaseline function will perform significance testing between two grouped BASELINE PDFs, by region, and return a data.frame with the following information:

- region: The sequence region, such as cdr and fwr.
- test: The name of the two groups compared.
- pvalue: Two-sided p-value for the comparison.
- fdr: FDR corrected p-value.

testBaseline(grouped_1, groupBy="sample_id")

```
## region test   pvalue     fdr
## 1   cdr -1h != +7d 0.05019208 0.08610636
## 2   fwr -1h != +7d 0.08610636 0.08610636
```

Plot and compare selection scores for groups

plotBaselineSummary plots the mean and confidence interval of selection scores for the given groups. The idColumn argument specifies the field that contains identifiers of the groups of sequences. If there is a secondary field by which the sequences are grouped, this can be specified using the groupColumn. This secondary grouping can have a user-defined color palette passed into groupColors or can be separated into facets by setting the facetBy="group". The subsetRegions argument can be used to visualize selection of specific regions. Several examples utilizing these different parameters are provided below.

# Set sample and isotype colors
sample_colors <- c(-1h="seagreen", +7d="steelblue")
isotype_colors <- c(IGHM="darkorchid", IGHD="firebrick",
                  IGHG="seagreen", IGHA="steelblue")

# Plot mean and confidence interval by time-point
plotBaselineSummary(grouped_1, "sample_id")
# Plot selection scores by time-point and isotype for only CDR
plotBaselineSummary(grouped_2, "sample_id", "c_call", groupColors=isotype_colors, subsetRegions="cdr")

# Group by CDR/FWR and facet by isotype
plotBaselineSummary(grouped_2, "sample_id", "c_call", facetBy="group")
plotBaselineDensity plots the full Baseline PDF of selection scores for the given groups. The parameters are the same as those for plotBaselineSummary. However, rather than plotting the mean and confidence interval, the full density function is shown.

```
# Plot selection PDFs for a subset of the data
plotBaselineDensity(grouped_2, "c_call", groupColumn="sample_id", colorElement="group",
colorValues=sample_colors, sigmaLimits=c(-1, 1))
```
Editing a field in a Baseline object

If, for any reason, one needs to edit the existing values in a field in a Baseline object, one can do so via editBaseline. In the following example, we remove results related to IGHA in the relevant fields from grouped_2. When the input data is large and it takes a long time for calcBaseline to run, editBaseline could become useful when, for instance, one would like to exclude a certain sample or isotype, but would rather not re-run calcBaseline after removing that sample or isotype from the original input data.

```r
# Get indices of rows corresponding to IGHA in the field "db"
# These are the same indices also in the matrices in the fields "numbOfSeqs",
# "binomK", "binomN", "binomP", and "pdfs"
# In this example, there is one row of IGHA for each sample

dbIgMIndex <- which(grouped_2$db[["c_call"]]) == "IGHA"

grouped_2 <- editBaseline(grouped_2, "db", grouped_2$db[-dbIgMIndex, ])
grouped_2 <- editBaseline(grouped_2, "numbOfSeqs", grouped_2$numbOfSeqs[-dbIgMIndex, ])
grouped_2 <- editBaseline(grouped_2, "binomK", grouped_2$binomK[-dbIgMIndex, ])
grouped_2 <- editBaseline(grouped_2, "binomN", grouped_2$binomN[-dbIgMIndex, ])
grouped_2 <- editBaseline(grouped_2, "binomP", grouped_2$binomP[-dbIgMIndex, ])
grouped_2 <- editBaseline(grouped_2, "pdfs",
  lapply(grouped_2$pdfs, function(pdfs) {pdfs[-dbIgMIndex, ]}))

# The indices corresponding to IGHA are slightly different in the field "stats"
# In this example, there is one row of IGHA for each sample and for each region

grouped_2 <- editBaseline(grouped_2, "stats",
  grouped_2$stats[grouped_2$stats[["c_call"]]) != "IGHA", ])
```