Intermediate *R* / *Bioconductor* for Sequence Analysis

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Chapter 1

Introduction

Intermediate *R* / *Bioconductor* for High-Throughput Sequence Analysis introduces users with some *R* experience to common *Bioconductor* work flows for sequence analysis. The course involves a combination of presentations and hands-on exercises. Our starting point is BAM files created by aligning short reads to a reference genome. Topics include: exploratory analysis (*GenomicRanges*, *Rsamtools*); assessing differential expression of known genes (*DESeq*); detection, calling, and manipulation of variants (*VariantTools*, *VariantAnnotation*). We learn how to integrate results with curated gene and genomic annotations (*GenomicFeatures*), and to visualize results (*GViz*, *ggbio*).

Course participants will have access to a configured Amazon machine instance, with easy access through a web browser and *Rstudio*; the only requirement is that users have *wireless internet capabilities* and have a modern web browser installed[1]. Participants wanting to use a version of *Bioconductor* on their own laptop should come with the 'devel' (to be *R* 3.0.0) version of *R* installed, and should have followed the package installation instructions available from the course web page[2] shortly before (e.g., Wednesday morning) the start of the course. Software installation will require high-speed internet access; relevant software can be installed for take-home use during the course.

There are many books to help with using *R*, but not yet a book-length treatment of *R* / *Bioconductor* tools for sequence analysis. Starting points for bioinformatic analysis in *R*, still relevant for statistical and informatic concepts though not directly addressing sequence analysis, are Hahne's *Bioconductor Case Studies* [9] and Gentleman's *R Programming for Bioinformatics* [8]. For *R* novices, one place to start is Pardis' *R for Beginners*[3]. General *R* programming recommendations include Dalgaard's *Introductory Statistics with R* [7], Matloff's *The Art of R Programming* [16], and Meys and de Vies' *R For Dummies* [17]; an interesting internet resource for intermediate *R* programming is Burns' *The R Inferno*[4].

[1] See requirements at`http://www.rstudio.com/ide/docs/advanced/optimizing_browser`

[2] `http://bioconductor.org/help/course-materials/2013/SeattleFeb2013/`

[3] `http://cran.r-project.org/doc/contrib/Paradis-rdebuts_en.pdf`

[4] `http://www.burns-stat.com/documents/books/the-r-inferno/`

Table 1.1: Tentative schedule.

Day 1	
Morning	Part I: Up to speed with *R* scripts and *Bioconductor* packages.
Afternoon	Part II: Differential expression.
Day 2	
Morning	Part III: Variants.
Afternoon	Part IV: Annotation and Visualization.

Part I

R / Bioconductor

Chapter 2

R

2.1 Statistical analysis and comprehension

R is an open-source statistical programming language. It is used to manipulate data, to perform statistical analysis, and to present graphical and other results. *R* consists of a core language, additional 'packages' distributed with the *R* language, and a very large number of packages contributed by the broader community. Packages add specific functionality to an *R* installation. *R* has become the primary language of academic statistical analysis, and is widely used in diverse areas of research, government, and industry.

R has several unique features. It has a surprisingly 'old school' interface: users type commands into a console; scripts in plain text represent work flows; tools other than *R* are used for editing and other tasks. *R* is a flexible programming language, so while one person might use functions provided by *R* to accomplish advanced analytic tasks, another might implement their own functions for novel data types. As a programming language, *R* adopts syntax and grammar that differ from many other languages: objects in *R* are 'vectors', and functions are 'vectorized' to operate on all elements of the object; *R* objects have 'copy on change' and 'pass by value' semantics, reducing unexpected consequences for users at the expense of less efficient memory use; common paradigms in other languages, such as the 'for' loop, are encountered much less commonly in *R*. Many authors contribute to *R*, so there can be a frustrating inconsistency of documentation and interface. *R* grew up in the academic community, so authors have not shied away from trying new approaches. Common statistical analysis functions are very well-developed.

2.2 Basics of *R*

Opening an *R* session results in a prompt. The user types instructions at the prompt. Here is an example:

```
> ## assign values 5, 4, 3, 2, 1 to variable 'x'
> x <- c(5, 4, 3, 2, 1)
> x

[1] 5 4 3 2 1
```

The first line starts with a # to represent a comment; the line is ignored by *R*. The next line creates a variable `x`. The variable is assigned (using `<-`, we could have used `=` almost interchangeably) a value. The value assigned is the result of a call to the `c` function. That it is a function call is indicated by the symbol named followed by parentheses, `c()`. The `c` function takes zero or more arguments, and returns a vector. The vector is the value assigned to `x`. *R* responds to this line with a new prompt, ready for the next input. The next line asks *R* to display the value of the variable `x`. *R* responds by printing `[1]` to indicate that the subsequent number is the first element of the vector. It then prints the value of `x`.

Table 2.1: Essential aspects of the *R* language.

Category	Function	Description
Vectors	`integer`, `numeric`	Vectors holding a single type of data length 0 or more
	`complex`, `character`	
	`raw`, `factor`	
List-like	`list`	Arbitrary collections of elements
	`data.frame`	List of equal-length vectors
	`environment`	*Pass-by-reference* data storage; hash
Array-like	`matrix`	Two-dimensional, homogeneous types
	`data.frame`	Homogeneous columns; row- and column indexing
	`array`	0 or more dimensions
Statistical	`NA`, `factor`	Essential statistical concepts, integral to the language.
Classes	'S3'	List-like structured data; simple inheritance & dispatch
	'S4'	Formal classes, multiple inheritance & dispatch
Functions	'function'	A simple function with arguments, body, and return value
	'generic'	A (S3 or S4) function with associated *methods*
	'method'	A function implementing a generic for an S3 or S4 class

R has many features to aid common operations. Entering sequences is a very common operation, and expressions of the form `2:4` create a sequence from `2` to `4`. Sub-setting one vector by another is enabled with `[`. Here we create an integer sequence from 2 to 4, and use the sequence as an index to select the second, third, and fourth elements of `x`

```
> x[2:4]
```

```
[1] 4 3 2
```

Index values can be repeated, and if outside the domain of `x` return the special value `NA`. Negative index values remove elements from the vector. Logical and character vectors (described below) can also be used for sub-setting.

R functions operate on variables. Functions are usually vectorized, acting on all elements of their argument and obviating the need for explicit iteration. Functions can generate warnings when performing suspect operations, or errors if evaluation cannot proceed; try `log(-1)`.

```
> log(x)
```

```
[1] 1.6094379 1.3862944 1.0986123 0.6931472 0.0000000
```

2.2.1 Essential data types

R has a number of built-in data types, summarized in Table 2.1. These represent `integer`, `numeric` (floating point), `complex`, `character`, `logical` (Boolean), and `raw` (byte) data. It is possible to convert between data types, and to discover the type or mode of a variable.

```
> c(1.1, 1.2, 1.3)         # numeric
```

```
[1] 1.1 1.2 1.3
```

```
> c(FALSE, TRUE, FALSE)    # logical
```

```
[1] FALSE  TRUE FALSE
```

```
> c("foo", "bar", "baz")   # character, single or double quote ok
```

```
[1] "foo" "bar" "baz"
```

```
> as.character(x)          # convert 'x' to character
```

```
[1] "5" "4" "3" "2" "1"
```

```
> typeof(x)                # the number 5 is numeric, not integer
```

```
[1] "double"
```

```
> typeof(2L)               # append 'L' to force integer
```

```
[1] "integer"
```

```
> typeof(2:4)              # ':' produces a sequence of integers
```

```
[1] "integer"
```

R includes data types particularly useful for statistical analysis, including `factor` to represent categories and `NA` (used in any vector) to represent missing values.

```
> sex <- factor(c("Male", "Female", NA), levels=c("Female", "Male"))
> sex
```

```
[1] Male   Female <NA>
Levels: Female Male
```

Lists, data frames, and matrices All of the vectors mentioned so far are homogeneous, consisting of a single type of element. A `list` can contain a collection of different types of elements and, like all vectors, these elements can be named to create a key-value association.

```
> lst <- list(a=1:3, b=c("foo", "bar"), c=sex)
> lst
```

```
$a
[1] 1 2 3

$b
[1] "foo" "bar"

$c
[1] Male   Female <NA>
Levels: Female Male
```

Lists can be subset like other vectors to get another list, or subset with `[[` to retrieve the actual list element; as with other vectors, sub-setting can use names

```
> lst[c(3, 1)]             # another list
```

```
$c
[1] Male   Female <NA>
Levels: Female Male

$a
[1] 1 2 3
```

```
> lst[["a"]]               # the element itself, selected by name
```

```
[1] 1 2 3
```

A `data.frame` is a list of equal-length vectors, representing a rectangular data structure not unlike a spread sheet. Each column of the data frame is a vector, so data types must be homogeneous within a column. A `data.frame` can be subset by row or column, and columns can be accessed with `$` or `[[`.

```
> df <- data.frame(age=c(27L, 32L, 19L),
+                  sex=factor(c("Male", "Female", "Male")))
> df
```

```
  age    sex
1  27   Male
2  32 Female
3  19   Male
```

```
> df[c(1, 3),]
```

```
  age  sex
1  27 Male
3  19 Male
```

```
> df[df$age > 20,]
```

```
  age    sex
1  27   Male
2  32 Female
```

A `matrix` is also a rectangular data structure, but subject to the constraint that all elements are the same type. A matrix is created by taking a vector, and specifying the number of rows or columns the vector is to represent.

```
> m <- matrix(1:12, nrow=3)
> m
```

```
     [,1] [,2] [,3] [,4]
[1,]    1    4    7   10
[2,]    2    5    8   11
[3,]    3    6    9   12
```

```
> m[c(1, 3), c(2, 4)]
```

```
     [,1] [,2]
[1,]    4   10
[2,]    6   12
```

On sub-setting, *R* coerces a single column `data.frame` or single row or column `matrix` to a vector if possible; use `drop=FALSE` to stop this behavior.

```
> m[, 3]
```

```
[1] 7 8 9
```

```
> m[, 3, drop=FALSE]
```

```
     [,1]
[1,]    7
[2,]    8
[3,]    9
```

An `array` is a data structure for representing homogeneous, rectangular data in higher dimensions.

2.2.2 S3 (and S4) classes

More complicated data structures are represented using the 'S3' or 'S4' object system. Objects are often created by functions (for example, `lm`, below), with parts of the object extracted or assigned using *accessor* functions. The following generates 1000 random normal deviates as `x`, and uses these to create another 1000 deviates `y` that are linearly related to `x` but with some error. We fit a linear regression using a 'formula' to describe the relationship between variables, summarize the results in a familiar ANOVA table, and access `fit` (an S3 object) for the residuals of the regression, using these as input first to the `var` (variance) and then `sqrt` (square-root) functions. Objects can be interrogated for their class.

```
> x <- rnorm(1000, sd=1)
> y <- x + rnorm(1000, sd=.5)
> fit <- lm(y ~ x)       # formula describes linear regression
> fit                    # an 'S3' object

Call:
lm(formula = y ~ x)

Coefficients:
(Intercept)            x
    0.01298      0.99425

> anova(fit)

Analysis of Variance Table

Response: y
           Df Sum Sq Mean Sq F value    Pr(>F)
x           1 921.92  921.92  3543.5 < 2.2e-16 ***
Residuals 998 259.65    0.26
---
Signif. codes:  0 âĂŸ***âĂŹ 0.001 âĂŸ**âĂŹ 0.01 âĂŸ*âĂŹ 0.05 âĂŸ.âĂŹ 0.1 âĂŸ âĂŹ 1

> sqrt(var(resid(fit)))  # residuals accessor and subsequent transforms

[1] 0.5098179

> class(fit)

[1] "lm"
```

Many *Bioconductor* packages implement S4 objects to represent data. S3 and S4 systems are quite different from a programmer's perspective, but fairly similar from a user's perspective: both systems encapsulate complicated data structures, and allow for methods specialized to different data types; accessors are used to extract information from the objects.

2.2.3 Functions

R has a very large number of functions; Table 2.2 provides a brief list of those that might be commonly used and particularly useful. See the help pages (e.g., `?lm`) and examples (`example(match)`) for more details on each of these functions.

R functions accept arguments, and return values. Arguments can be required or optional. Some functions may take variable numbers of arguments, e.g., the columns in a `data.frame`

Table 2.2: A selection of *R* function.

`dir`, `read.table` **(and friends)**, `scan` List files in a directory, read spreadsheet-like data into *R*, efficiently read homogeneous data (e.g., a file of numeric values) to be represented as a matrix.

`c`, `factor`, `data.frame`, `matrix` Create a vector, factor, data frame or matrix.

`summary`, `table`, `xtabs` Summarize, create a table of the number of times elements occur in a vector, cross-tabulate two or more variables.

`t.test`, `aov`, `lm`, `anova`, `chisq.test` Basic comparison of two (`t.test`) groups, or several groups via analysis of variance / linear models (`aov` output is probably more familiar to biologists), or compare simpler with more complicated models (`anova`); χ^2 tests.

`dist`, `hclust` Cluster data.

`plot` Plot data.

`ls`, `str`, `library`, `search` List objects in the current (or specified) workspace, or peak at the structure of an object; add a library to or describe the search path of attached packages.

`lapply`, `sapply`, `mapply`, `aggregate` Apply a function to each element of a list (`lapply`, `sapply`), to elements of several lists (`mapply`), or to elements of a list partitioned by one or more factors (`aggregate`).

`with` Conveniently access columns of a data frame or other element without having to repeat the name of the data frame.

`match`, `%in%` Report the index or existence of elements from one vector that match another.

`split`, `cut`, `unlist` Split one vector by an equal length factor, cut a single vector into intervals encoded as levels of a factor, unlist (concatenate) list elements.

`strsplit`, `grep`, `sub` Operate on character vectors, splitting it into distinct fields, searching for the occurrence of a patterns using regular expressions (see `?regex`, or substituting a string for a regular expression.

`install.packages` Install a package from an on-line repository into your *R*.

`traceback`, `debug`, `browser` Report the sequence of functions under evaluation at the time of the error; enter a debugger when a particular function or statement is invoked.

```
> y <- 5:1
> log(y)

[1] 1.6094379 1.3862944 1.0986123 0.6931472 0.0000000

> args(log)        # arguments 'x' and 'base'; see ?log

function (x, base = exp(1))
NULL

> log(y, base=2)   # 'base' is optional, with default value

[1] 2.321928 2.000000 1.584963 1.000000 0.000000

> try(log())       # 'x' required; 'try' continues even on error
> args(data.frame) # ... represents variable number of arguments

function (..., row.names = NULL, check.rows = FALSE, check.names = TRUE,
    stringsAsFactors = default.stringsAsFactors())
NULL
```

Arguments can be matched by name or position. If an argument appears after `...`, it must be named.

```
> log(base=2, y)   # match argument 'base' by name, 'x' by position

[1] 2.321928 2.000000 1.584963 1.000000 0.000000
```

A function such as `anova` is a *generic* that provides an overall signature but dispatches the actual work to the *method* corresponding to the class(es) of the arguments used to invoke the generic. A generic may have fewer arguments than a method, as with the S3 function `anova` and its method `anova.glm`.

```
> args(anova)

function (object, ...)
NULL

> args(anova.glm)

function (object, ..., dispersion = NULL, test = NULL)
NULL
```

The `...` argument in the `anova` generic means that additional arguments are possible; the `anova` generic hands these arguments to the method it dispatches to.

Exercise 1
This exercise uses data describing 128 microarray samples as a basis for exploring R functions. Covariates such as age, sex, type, stage of the disease, etc., are in a data file `pData.csv`*.*

The following command creates a variable `pdataFiles` *that is the location of a comma-separated value ('csv') file to be used in the exercise. A csv file can be created using, e.g., 'Save as...' in spreadsheet software.*

```
> pdataFile <- system.file(package="SequenceAnalysisData", "extdata",
+                          "pData.csv")
```

Input the csv file using `read.table`, assigning the input to a variable `pdata`. Use `dim` to find out the dimensions (number of rows, number of columns) in the object. Are there 128 rows? Use `names` or `colnames` to list the names of the columns of `pdata`. Use `summary` to summarize each column of the data. What are the data types of each column in the data frame?

A data frame is a list of equal length vectors. Select the 'sex' column of the data frame using `[[` or `$`. Pause to explain to your neighbor why this sub-setting works. Since a data frame is a list, use `sapply` to ask about the class of each column in the data frame. Explain to your neighbor what this produces, and why.

Use `table` to summarize the number of males and females in the sample. Consult the help page `?table` to figure out additional arguments required to include `NA` values in the tabulation.

The `mol.biol` column summarizes molecular biological attributes of each sample. Use `table` to summarize the different molecular biology levels in the sample. Use `%in%` to create a logical vector of the samples that are either `BCR/ABL` or `NEG`. Subset the original phenotypic data to contain those samples that are `BCR/ABL` or `NEG`.

After sub-setting, what are the levels of the `mol.biol` column? Set the levels to be `BCR/ABL` and `NEG`, i.e., the levels in the subset.

One would like covariates to be similar across groups of interest. Use `t.test` to assess whether `BCR/ABL` and `NEG` have individuals with similar age. To do this, use a `formula` that describes the response `age` in terms of the predictor `mol.biol`. If age is not independent of molecular biology, what complications might this introduce into subsequent analysis? Use

Solution: Here we input the data and explore basic properties.

```
> pdata <- read.table(pdataFile)
> dim(pdata)
```

```
[1] 128  21
```

```
> names(pdata)
```

```
 [1] "cod"             "diagnosis"       "sex"             "age"            
 [5] "BT"              "remission"       "CR"              "date.cr"        
 [9] "t.4.11."         "t.9.22."         "cyto.normal"     "citog"          
[13] "mol.biol"        "fusion.protein"  "mdr"             "kinet"          
[17] "ccr"             "relapse"         "transplant"      "f.u"            
[21] "date.last.seen"
```

```
> summary(pdata)
```

```
      cod           diagnosis     sex          age              BT    
 10005  :  1   1/15/1997 :  2   F   :42   Min.   : 5.00   B2     :36  
 1003   :  1   1/29/1997 :  2   M   :83   1st Qu.:19.00   B3     :23  
 1005   :  1   11/15/1997:  2   NA's: 3   Median :29.00   B1     :19  
 1007   :  1   2/10/1998 :  2             Mean   :32.37   T2     :15  
 1010   :  1   2/10/2000 :  2             3rd Qu.:45.50   B4     :12  
 11002  :  1   (Other)   :116             Max.   :58.00   T3     :10  
 (Other):122   NA's      :  2             NA's   :5       (Other):13  
 remission                  CR           date.cr    t.4.11.       
 CR  :99   CR                :96   11/11/1997: 3   Mode :logical  
 REF :15   DEATH IN CR       : 3   1/21/1998 : 2   FALSE:86       
 NA's:14   DEATH IN INDUCTION: 7   10/18/1999: 2   TRUE :7        
           REF               :15   12/7/1998 : 2   NA's :35       
           NA's              : 7   1/17/1997 : 1                  
                                   (Other)   :87                  
                                   NA's      :31
  t.9.22.        cyto.normal               citog        mol.biol
 Mode :logical   Mode :logical   normal       :24   ALL1/AF4:10
 FALSE:67        FALSE:69        simple alt.  :15   BCR/ABL :37
 TRUE :26        TRUE :24        t(9;22)      :12   E2A/PBX1: 5
 NA's :35        NA's :35        t(9;22)+other:11   NEG     :74
                                 complex alt. :10   NUP-98  : 1
                                 (Other)      :21   p15/p16 : 1
                                 NA's         :35
   fusion.protein   mdr          kinet       ccr           relapse
 p190     :17     NEG :101   dyploid:94   Mode :logical   Mode :logical
 p190/p210: 8     POS : 24   hyperd.:27   FALSE:74        FALSE:35
 p210     : 8     NA's:  3   NA's   : 7   TRUE :26        TRUE :65
 NA's     :95                             NA's :28        NA's :28



 transplant                     f.u        date.last.seen
 Mode :logical   REL                :61   1/7/1998  : 2
 FALSE:91        CCR                :23   12/15/1997: 2
 TRUE :9         BMT / DEATH IN CR: 4   12/31/2002: 2
 NA's :28        BMT / CCR          : 3   3/29/2001 : 2
                 DEATH IN CR        : 2   7/11/1997 : 2
                 (Other)            : 7   (Other)   :83
                 NA's               :28   NA's      :35
```

A data frame can be subset as if it were a matrix, or a list of column vectors.

```
> head(pdata[,"sex"], 3)
```

```
[1] M M F
Levels: F M
```

```
> head(pdata$sex, 3)
```

```
[1] M M F
Levels: F M
```

```
> head(pdata[["sex"]], 3)
```

```
[1] M M F
Levels: F M
```

```
> sapply(pdata, class)
```

```
           cod      diagnosis            sex            age             BT
      "factor"       "factor"       "factor"      "integer"       "factor"
     remission             CR        date.cr       t.4.11.       t.9.22.
      "factor"       "factor"       "factor"      "logical"      "logical"
   cyto.normal          citog       mol.biol fusion.protein            mdr
     "logical"       "factor"       "factor"       "factor"       "factor"
         kinet            ccr        relapse     transplant            f.u
      "factor"      "logical"      "logical"      "logical"       "factor"
date.last.seen
      "factor"
```

The number of males and females, including NA, is

```
> table(pdata$sex, useNA="ifany")

   F    M <NA>
  42   83    3
```

An alternative version of this uses the with function: with(pdata, table(sex, useNA="ifany")).

The mol.biol column contains the following samples:

```
> with(pdata, table(mol.biol, useNA="ifany"))

mol.biol
ALL1/AF4  BCR/ABL E2A/PBX1      NEG   NUP-98  p15/p16
      10       37        5       74        1        1
```

A logical vector indicating that the corresponding row is either BCR/ABL or NEG is constructed as

```
> ridx <- pdata$mol.biol %in% c("BCR/ABL", "NEG")
```

We can get a sense of the number of rows selected via table or sum (discuss with your neighbor what sum does, and why the answer is the same as the number of TRUE values in the result of the table function).

```
> table(ridx)

ridx
FALSE  TRUE
   17   111

> sum(ridx)

[1] 111
```

The original data frame can be subset to contain only BCR/ABL or NEG samples using the logical vector ridx that we created.

```
> pdata1 <- pdata[ridx,]
```

The levels of each factor reflect the levels in the original object, rather than the levels in the subset object, e.g.,

```
> levels(pdata1$mol.biol)

[1] "ALL1/AF4" "BCR/ABL"  "E2A/PBX1" "NEG"      "NUP-98"   "p15/p16"
```

These can be re-coded by updating the new data frame to contain a factor with the desired levels.

```
> pdata1$mol.biol <- factor(pdata1$mol.biol)
> table(pdata1$mol.biol)

BCR/ABL     NEG
     37      74
```

To ask whether age differs between molecular biology types, we use a formula age ~ mol.biol to describe the relationship (‘age as a function of molecular biology’) that we wish to test

```
> with(pdata1, t.test(age ~ mol.biol))
```

Table 2.3: Tools for debugging and error-handling.

Function	Description
`traceback`	Report the 'call stack' at the time of an error.
`options(error=)`	Set a handler to be executed on error, e.g., `error=recover`.
`debug`, `trace`	Enter the browser when a function is called
`browser`	Interactive debugging
`tryCatch`	Handle an error condition in a script.

```
        Welch Two Sample t-test

data:  age by mol.biol
t = 4.8172, df = 68.529, p-value = 8.401e-06
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  7.13507 17.22408
sample estimates:
mean in group BCR/ABL     mean in group NEG
             40.25000              28.07042
```

This summary can be visualize with, e.g., the `boxplot` function

```
> ## not evaluated
> boxplot(age ~ mol.biol, pdata1)
```

Molecular biology seem to be strongly associated with age; individuals in the `NEG` group are considerably younger than those in the `BCR/ABL` group. We might wish to include age as a covariate in any subsequent analysis seeking to relate molecular biology to gene expression.

2.3 In and out of trouble

2.3.1 Warnings, errors, and debugging

R signals unexpected results through warnings and errors. Warnings occur when the calculation produces an unusual result that nonetheless does not preclude further evaluation. For instance `log(-1)` results in a value `NaN` ('not a number') that allows computation to continue, but at the same time signals a warning

```
> log(-1)
[1] NaN
Warning message:
In log(-1) : NaNs produced
```

Errors result when the inputs or outputs of a function are such that no further action can be taken, e.g., trying to take the square root of a character vector

```
> sqrt("two")
Error in sqrt("two") : Non-numeric argument to mathematical function
```

Warnings and errors occurring at the command prompt are usually easy to diagnose. They can be more enigmatic when occurring in a function, and exacerbated by sometimes cryptic (when read out of context) error messages. Some key tools for figuring out ('debugging') errors are summarized in Table 2.3.

An initial step in coming to terms with errors is to simplify the problem as much as possible, aiming for a 'reproducible' error. The reproducible error might involve a very small (even trivial) data set that immediately provokes the error. Often the process of creating a reproducible example helps to clarify what the error is, and what possible solutions might be.

Invoking `traceback()` immediately after an error occurs provides a 'stack' of the function calls that were in effect when the error occurred. This can help understand the context in which the error occurred. Knowing the context, one might use `debug` (or its more elaborate cousin, `trace`) to enter into a browser (see `?browser`) that allows one to step through the function in which the error occurred.

It can sometimes be useful to use global options (see `?options`) to influence what happens when an error occurs. Two common global options are `error` and `warn`. Setting `error=recover` combines the functionality of `traceback` and `debug`, allowing the user to enter the browser at any level of the call stack in effect at the time the error occurred. Default error behavior can be restored with `options(error=NULL)`. Setting `warn=2` causes warnings to be promoted to errors. For instance, initial investigation of an error might show that the error occurs when one of the arguments to a function has value `NaN`. The error might be accompanied by a warning message that the `NaN` has been introduced, but because warnings are by default not reported immediately it is not clear where the `NaN` comes from. `warn=2` means that the warning is treated as an error, and hence can be debugged using `traceback`, `debug`, and so on.

It is possible to continue evaluation even after an error occurs. The simplest mechanism uses the `try` function, but an only slightly more complicated version providing greater flexibility it `tryCatch`. `tryCatch` allows one to write a `handler` (the `error` argument to `tryCatch`, below) to address common faults in a way that allows a script to continue executing. Suppose a function `f` fails under certain conditions

```
> f <- function(i) {
+     if (i < 0)
+         stop("i is negative")
+     rnorm(i)
+ }
> lapply(0:1, f)
```

```
[[1]]
numeric(0)

[[2]]
[1] 0.8270607
```

but we wish to continue, e.g., replacing failed conditions with `NA`:

```
> lapply(-1:1, function(i) {
+     tryCatch({
+         f(i)
+     }, error=function(err) {
+         ## return 'NA' when error occurs, instead of stopping
+         NA_real_
+     })
+ })
```

```
[[1]]
[1] NA

[[2]]
numeric(0)

[[3]]
[1] 0.8272511
```

Table 2.4: Common ways to improve efficiency of *R* code.

Easy	Moderate
1. Selective input	1. Know relevant packages
2. Vectorize	2. Understand algorithm complexity
3. Pre-allocate and fill	3. Use parallel evaluation
4. Avoid expensive conveniences	4. Exploit libraries and `C++` code

Table 2.5: Tools for measuring performance.

Function	Description
`identical`	Compare content of objects.
`all.equal`	
`system.time`	Time required to evaluate an expression
`Rprof`	Time spent in each function; also `summaryRprof`.
`tracemem`	Indicate when memory copies occur (*R* must be configured to support this).
rbenchmark	Packages for standardizing speed measurment
microbenchmark	

2.3.2 Efficient *R* code

There are often many ways to accomplish a result in *R*, but these different ways often have very different speed or memory requirements. For small data sets these performance differences are not that important, but for large data sets (e.g., high-throughput sequencing; genome-wide association studies, GWAS) or complicated calculations (e.g., bootstrapping) performance can be important. Several approaches to achieving efficient *R* programming are summarized in Table 2.4; common tools used to help with assessing performance (including comparison of results from different implementations!) are in Table 2.5.

Easy solutions Several common performance bottlenecks often have easy solutions; these are outlined here.

Text files often contain more information, for example 1000's of individuals at millions of SNPs, when only a subset of the data is required, e.g., during algorithm development. Reading in all the data can be demanding in terms of both memory and time. A solution is to use arguments such as `colClasses` to specify the columns and their data types that are required, and to use `nrow` to limit the number of rows input. For example, the following ignores the first and fourth column, reading in only the second and third (as type `integer` and `numeric`).

```
> ## not evaluated
> colClasses <-
+   c("NULL", "integer", "numeric", "NULL")
> df <- read.table("myfile", colClasses=colClasses)
```

R is vectorized, so traditional programming `for` loops are often not necessary. Rather than calculating 100000 random numbers one at a time, or squaring each element of a vector, or iterating over rows and columns in a matrix to calculate row sums, invoke the single function that performs each of these operations.

```
> x <- runif(100000); x2 <- x^2
> m <- matrix(x2, nrow=1000); y <- rowSums(m)
```

This often requires a change of thinking, turning the sequence of operations 'inside-out'. For instance, calculate the log of the square of each element of a vector by calculating the square of all elements, followed by the log of all elements `x2 <- x^2; x3 <- log(x2)`, or simply `logx2 <- log(x^2)`.

It may sometimes be natural to formulate a problem as a `for` loop, or the formulation of the problem may require that a `for` loop be used. In these circumstances the appropriate strategy is to pre-allocate the `result` object, and to fill the result in during loop iteration.

```
> ## not evaluated
> result <- numeric(nrow(df))
> for (i in seq_len(nrow(df)))
+   result[[i]] <- some_calc(df[i,])
```

Failure to pre-allocate and fill is the second cirle of *R* hell [5].

Some *R* operations are helpful in general, but misleading or inefficient in particular circumstances. An example is the behavior of `unlist` when the list is named – *R* creates new names that have been made unique. This can be confusing (e.g., when Entrez gene identifiers are 'mangled' to unintentionally look like other identifiers) and expensive (when a large number of new names need to be created). Avoid creating unnecessary names, e.g.,

```
> unlist(list(a=1:2)) # name 'a' becomes 'a1', 'a2'

a1 a2
 1  2

> unlist(list(a=1:2), use.names=FALSE)   # no names

[1] 1 2
```

Names can be very useful for avoiding book-keeping errors, but are inefficient for repeated look-ups; use vectorized access or numeric indexing.

Moderate solutions Several solutions to inefficient code require greater knowledge to implement.

Using appropriate functions can greatly influence performance; it takes experience to know when an appropriate function exists. For instance, the `lm` function could be used to assess differential expression of each gene on a microarray, but the *limma* package implements this operation in a way that takes advantage of the experimental design that is common to each probe on the microarray, and does so in a very efficient manner.

```
> ## not evaluated
> library(limma) # microarray linear models
> fit <- lmFit(eSet, design)
```

Using appropriate algorithms can have significant performance benefits, especially as data becomes larger. This solution requires moderate skills, because one has to be able to think about the complexity (e.g., expected number of operations) of an algorithm, and to identify algorithms that accomplish the same goal in fewer steps. For example, a naive way of identifying which of 100 numbers are in a set of size 10 might look at all 100 × 10 combinations of numbers (i.e., polynomial time), but a faster way is to create a 'hash' table of one of the set of elements and probe that for each of the other elements (i.e., linear time). The latter strategy is illustrated with

```
> x <- 1:100; s <- sample(x, 10)
> inS <- x %in% s
```

R supports parallel evaluation, most easily through the `mclapply` function of the *parallel* package distributed with base *R* (`mclapply` is unfortunately not available on Windows). Parallel evaluation is discussed further in Chapter 7.

R is an interpreted language, and for very challenging computational problems it may be appropriate to write critical stages of an analysis in a compiled language like C or Fortran, or to use an existing programming library (e.g., the BOOST library) that efficiently implements advanced algorithms. *R* has a well-developed interface to C or Fortran, so it is 'easy' to do this; the *Rcpp* package provides a very nice approach for those familiar with C++ concepts. This places a significant burden on the person implementing the solution, requiring knowledge of two or more computer languages and of the interface between them.

Measuring performance When trying to improve performance, one wants to ensure (a) that the new code is actually faster than the previous code, and (b) both solutions arrive at the same, correct, answer.

The `system.time` function is a straight-forward way to measure the length of time a portion of code takes to evaluate.

```
> m <- matrix(runif(200000), 20000)
> system.time(apply(m, 1, sum))
```

```
   user  system elapsed 
  0.092   0.008   0.098 
```

When comparing performance of different functions, it is appropriate to replicate timings to average over vagaries of system use, and to shuffle the order in which timings of alternative algorithms are calculated to avoid artifacts such as initial memory allocation. Rather than creating *ad hoc* approaches to timing, it is convenient to use packages such as *rbenchmark*:

```
> library(rbenchmark)
> f0 <- function(x) apply(x, 1, sum)
> f1 <- function(x) rowSums(x)
> benchmark(f0(m), f1(m),
+           columns=c("test", "elapsed", "relative"),
+           replications=5)
```

```
   test elapsed relative
1 f0(m)   0.608  202.667
2 f1(m)   0.003    1.000
```

Speed is an important metric, but equivalent results are also needed. The functions `identical` and `all.equal` provide different levels of assessing equivalence, with `all.equal` providing ability to ignore some differences, e.g., in the names of vector elements.

```
> res1 <- apply(m, 1, sum)
> res2 <- rowSums(m)
> identical(res1, res2)
```

```
[1] TRUE
```

```
> identical(c(1, -1), c(x=1, y=-1))
```

```
[1] FALSE
```

```
> all.equal(c(1, -1), c(x=1, y=-1),
+           check.attributes=FALSE)
```

```
[1] TRUE
```

Two additional functions for assessing performance are `Rprof` and `tracemem`; these are mentioned only briefly here. The `Rprof` function profiles *R* code, presenting a summary of the time spent in each part of several lines of *R* code. It is useful for gaining insight into the location of performance bottlenecks when these are not readily apparent from direct inspection. Memory management, especially copying large objects, can frequently contribute to poor performance. The `tracemem` function allows one to gain insight into how *R* manages memory; insights from this kind of analysis can sometimes be useful in restructuring code into a more efficient sequence.

Table 2.6: Selected base and contributed packages.

Package	Description
base	Data input and manipulation; scripting and programming.
stats	Essential statistical and plotting functions.
lattice, *ggplot2*	Approaches to advanced graphics.
methods	‘S4’ classes and methods.
parallel	Facilities for parallel evaluation.

2.4 Packages

Packages provide functionality beyond that available in base *R*. There are over 4000 packages in CRAN (comprehensive *R* archive network) and more than 670 *Bioconductor* packages. Packages are contributed by diverse members of the community; they vary in quality (many are excellent) and sometimes contain idiosyncratic aspects to their implementation. Table 2.6 outlines key base packages and selected contributed packages; see a local CRAN mirror (including the task views summarizing packages in different domains) and *Bioconductor* for additional contributed packages.

The *lattice* package illustrates the value packages add to base *R*. *lattice* is distributed with *R* but not loaded by default. It provides a very expressive way to visualize data. The following example plots yield for a number of barley varieties, conditioned on site and grouped by year. Figure 2.1 is read from the lower left corner. Note the common scales, efficient use of space, and not-too-pleasing default color palette. The Morris sample appears to be mis-labeled for ‘year’, an apparent error in the original data. Find out about the built-in data set used in this example with `?barley`.

```
> library(lattice)
> plt <- dotplot(variety ~ yield | site, data = barley, groups = year,
+                xlab = "Barley Yield (bushels/acre)" , ylab=NULL,
+                key = simpleKey(levels(barley$year), space = "top",
+                  columns=2),
+                aspect=0.5, layout = c(2,3))
> print(plt)
```

New packages (from *Bioconductor* or CRAN) can be added to an *R* installation using `biocLite()`:

```
source("http://bioconductor.org/biocLite.R")
biocLite(c("GenomicRanges", "ShortRead"))
```

A package is installed only once per *R* installation, but needs to be loaded (with `library`) in each session in which it is used. Loading a package also loads any package that it depends on. Packages loaded in the current session are displayed with `search`. The ordering of packages returned by `search` represents the order in which the global environment (where commands entered at the prompt are evaluated) and attached packages are searched for symbols.

```
> length(search())
```

```
[1] 21
```

```
> search()
```

```
 [1] ".GlobalEnv"                  "package:lattice"
 [3] "package:rbenchmark"          "package:SequenceAnalysisData"
 [5] "package:edgeR"               "package:limma"
 [7] "package:GenomicFeatures"     "package:AnnotationDbi"
 [9] "package:Biobase"             "package:GenomicRanges"
```

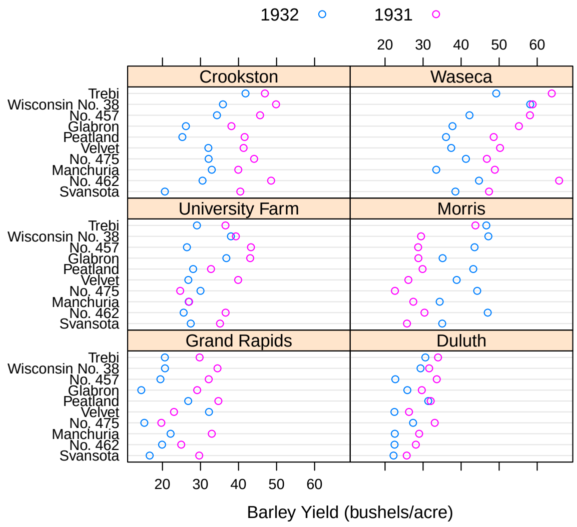


Figure 2.1: Variety yield conditional on site and grouped by year, for the `barley` data set. What's up with Morris?

```
[11] "package:IRanges"              "package:BiocGenerics"
[13] "package:parallel"             "package:stats"
[15] "package:graphics"             "package:grDevices"
[17] "package:utils"                "package:datasets"
[19] "package:methods"              "Autoloads"
[21] "package:base"
```

It is possible for a package earlier in the search path to mask symbols later in the search path; these can be disambiguated using `::`.

```
> base::log(1:3)
```

```
[1] 0.0000000 0.6931472 1.0986123
```

Exercise 2
Use the `library` function to load the IntermediateSequenceAnalysis2013 package. Use the `sessionInfo` function to verify that you are using R version 2.15.2 and current packages, similar to those reported here. What other packages were loaded along with IntermediateSequenceAnalysis2013?

Solution:

```
> library(IntermediateSequenceAnalysis2013)
> sessionInfo()
```

2.5 Help!

Find help using the *R* help system. Start a web browser with

```
> help.start()
```

The ‘Search Engine and Keywords’ link is helpful in day-to-day use.

Manual pages Use manual pages to find detailed descriptions of the arguments and return values of functions, and the structure and methods of classes. Find help within an *R* session as

```
> ?data.frame
> ?lm
> ?anova             # a generic function
> ?anova.lm          # an S3 method, specialized for 'lm' objects
```

S3 methods can be queried interactively. For S3,

```
> methods(anova)

[1] anova.MAList  anova.glm     anova.glmlist anova.lm      anova.loess*
[6] anova.mlm     anova.nls*

   Non-visible functions are asterisked

> methods(class="glm")

 [1] add1.glm*           anova.glm           confint.glm*
 [4] cooks.distance.glm* deviance.glm*       drop1.glm*
 [7] effects.glm*        extractAIC.glm*     family.glm*
[10] formula.glm*        influence.glm*      logLik.glm*
[13] model.frame.glm     nobs.glm*           predict.glm
[16] print.glm           residuals.glm       rstandard.glm
[19] rstudent.glm        summary.glm         vcov.glm*
[22] weights.glm*

   Non-visible functions are asterisked
```

It is often useful to view a method definition, either by typing the method name at the command line or, for ‘non-visible’ methods, using `getAnywhere`:

```
> anova.lm
> getAnywhere("anova.loess")
```

For instance, the source code of a function is printed if the function is invoked without parentheses. Here we discover that the function `head` (which returns the first 6 elements of anything) defined in the *utils* package, is an S3 generic (indicated by `UseMethod`) and has several methods. We use `head` to look at the first six lines of the `head` method specialized for `matrix` objects.

```
> utils::head

function (x, ...)
UseMethod("head")
<environment: namespace:utils>

> methods(head)

[1] head.data.frame* head.default*    head.ftable*     head.function*
[5] head.matrix      head.table*

   Non-visible functions are asterisked
```

```
> head(head.matrix)

1 function (x, n = 6L, ...)
2 {
3     stopifnot(length(n) == 1L)
4     n <- if (n < 0L)
5         max(nrow(x) + n, 0L)
6     else min(n, nrow(x))
```

Vignettes Vignettes, especially in *Bioconductor* packages, provide an extensive narrative describing overall package functionality. Use

```
> vignette(package="IntermediateSequenceAnalysis2013")
```

to see, in your web browser, vignettes available in the *IntermediateSequenceAnalysis2013* package. Vignettes usually consist of text with embedded *R* code, a form of literate programming. The vignette can be read as a PDF document, while the *R* source code is present as a script file ending with extension `.R`. The script file can be sourced or copied into an *R* session to evaluate exactly the commands used in the vignette. For *Bioconductor* packages, vignettes are available on the package 'landing page', e.g., for *IRanges*[1]

[1] http://bioconductor.org/packages/devel/bioc/html/IRanges.html

Chapter 3

Bioconductor

Bioconductor is a collection of *R* packages for the analysis and comprehension of high-throughput genomic data. *Bioconductor* started more than 10 years ago, and is widely used (Figure 3.1. It gained credibility for its statistically rigorous approach to microarray pre-processing and analysis of designed experiments, and integrative and reproducible approaches to bioinformatic tasks. There are now more than 670 *Bioconductor* packages for expression and other microarrays, sequence analysis, flow cytometry, imaging, and other domains. The *Bioconductor* web site provides installation, package repository, help, and other documentation.

The *Bioconductor* web site is at `bioconductor.org`. Features include:

- Introductory work flows.
- A manifest of *Bioconductor* packages arranged in BiocViews.
- Annotation (data bases of relevant genomic information, e.g., Entrez gene ids in model organisms, KEGG pathways) and experiment data (containing relatively comprehensive data sets and their analysis) packages.
- Mailing lists, including searchable archives, as the primary source of help.
- Course and conference information, including extensive reference material.
- General information about the project.
- Package developer resources, including guidelines for creating and submitting new packages.

Exercise 3
Scavenger hunt. Spend five minutes tracking down the following information.

a. *From the Bioconductor web site, instructions for installing or updating Bioconductor packages.*

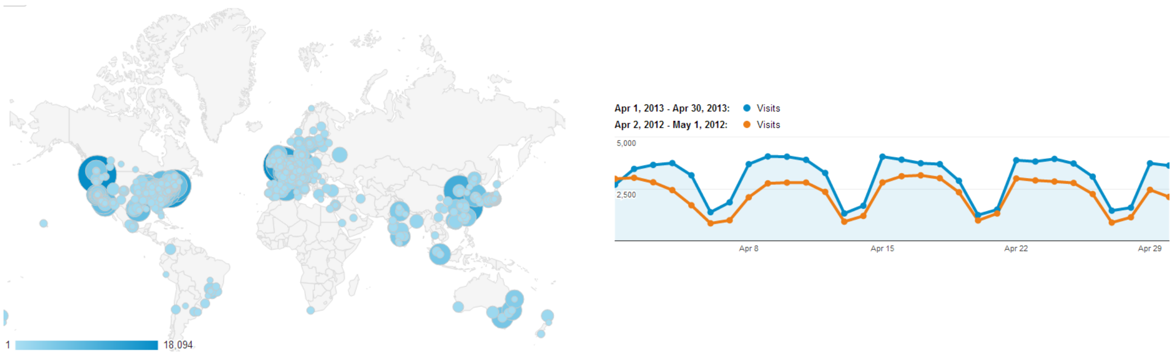


Figure 3.1: *Bioconductor* Google analytics, 1-month access, 10 December 2012. Left: access by country. Right: daily access in 2011 (orange) and 2012 (blue).

b. A list of all packages in the current release of Bioconductor.

c. The URL of the Bioconductor mailing list subscription page.

Solution: Possible solutions from the *Bioconductor* web site are, e.g., `http://bioconductor.org/install/` (installation instructions), `http://bioconductor.org/packages/release/bioc/` (current software packages), `http://bioconductor.org/help/mailing-list/` (mailing lists).

3.1 High-throughput sequence analysis

Recent technological developments introduce high-throughput sequencing approaches. A variety of experimental protocols and analysis work flows address gene expression, regulation, and encoding of genetic variants. Experimental protocols produce a large number (tens of millions per sample) of short (e.g., 35-150, single or paired-end) nucleotide sequences. These are aligned to a reference or other genome. Analysis work flows use the alignments to infer levels of gene expression (RNA-seq), binding of regulatory elements to genomic locations (ChIP-seq), or prevalence of structural variants (e.g., SNPs, short indels, large-scale genomic rearrangements). Sample sizes range from minimal replication (e.g,. 2 samples per treatment group) to thousands of individuals.

3.2 Statistical programming

Many academic and commercial software products are available; why would one use *R* and *Bioconductor*? One answer is to ask about the demands high-throughput genomic data places on effective computational biology software.

Effective computational biology software High-throughput questions make use of large data sets. This applies both to the primary data (microarray expression values, sequenced reads, etc.) and also to the annotations on those data (coordinates of genes and features such as exons or regulatory regions; participation in biological pathways, etc.). Large data sets place demands on our tools that preclude some standard approaches, such as spread sheets. Likewise, intricate relationships between data and annotation, and the diversity of research questions, require flexibility typical of a programming language rather than a narrowly-enabled graphical user interface.

Analysis of high-throughput data is necessarily statistical. The volume of data requires that it be appropriately summarized before any sort of comprehension is possible. The data are produced by advanced technologies, and these introduce artifacts (e.g., probe-specific bias in microarrays; sequence or base calling bias in RNA-seq experiments) that need to be accommodated to avoid incorrect or inefficient inference. Data sets typically derive from designed experiments, requiring a statistical approach both to account for the design and to correctly address the large number of observed values (e.g., gene expression or sequence tag counts) and small number of samples accessible in typical experiments.

Research needs to be reproducible. Reproducibility is both an ideal of the scientific method, and a pragmatic requirement. The latter comes from the long-term and multi-participant nature of contemporary science. An analysis will be performed for the initial experiment, revisited again during manuscript preparation, and revisited during reviews or in determining next steps. Likewise, analysis typically involve a team of individuals with diverse domains of expertise. Effective collaborations result when it is easy to reproduce, perhaps with minor modifications, an existing result, and when sophisticated statistical or bioinformatic analysis can be effectively conveyed to other group members.

Science moves very quickly. This is driven by the novel questions that are the hallmark of discovery, and by technological innovation and accessibility. Rapidity of scientific development places significant burdens on software, which must also move quickly. Effective software cannot be too polished, because that requires that the correct analyses are 'known' and that significant resources of time and money have been invested in developing the software; this implies software that is tracking the trailing edge of innovation. On the other hand, leading-edge software cannot be too idiosyncratic; it must be usable by a wider audience than the creator of the software, and fit in with other software relevant to the analysis.

Effective software must be accessible. Affordability is one aspect of accessibility. Another is transparent implementation, where the novel software is sufficiently documented and source code accessible enough for the assumptions, approaches, practical implementation decisions, and inevitable coding errors to be assessed by other skilled practitioners. A final aspect of affordability is that the software is actually usable. This is achieved through adequate documentation, support forums, and training opportunities.

***Bioconductor* as effective computational biology software** What features of *R* and *Bioconductor* contribute to its effectiveness as a software tool?

Bioconductor is well suited to handle extensive data and annotation. *Bioconductor* 'classes' represent high-throughput data and their annotation in an integrated way. *Bioconductor* methods use advanced programming techniques or *R* resources (such as transparent data base or network access) to minimize memory requirements and integrate with diverse resources. Classes and methods coordinate complicated data sets with extensive annotation. Nonetheless, the basic model for object manipulation in *R* involves vectorized in-memory representations. For this reason, particular programming paradigms (e.g., block processing of data streams; explicit parallelism) or hardware resources (e.g., large-memory computers) are sometimes required when dealing with extensive data.

R is ideally suited to addressing the statistical challenges of high-throughput data. Three examples include the development of the 'RMA' and other normalization algorithm for microarray pre-processing, use of moderated *t*-statistics for assessing microarray differential expression, and development of negative binomial approaches to estimating dispersion read counts necessary for appropriate analysis of RNAseq designed experiments.

Many of the 'old school' aspects of *R* and *Bioconductor* facilitate reproducible research. An analysis is often represented as a text-based script. Reproducing the analysis involves re-running the script; adjusting how the analysis is performed involves simple text-editing tasks. Beyond this, *R* has the notion of a 'vignette', which represents an analysis as a LaTeX document with embedded *R* commands. The *R* commands are evaluated when the document is built, thus reproducing the analysis. The use of LaTeX means that the symbolic manipulations in the script are augmented with textual explanations and justifications for the approach taken; these include graphical and tabular summaries at appropriate places in the analysis. *R* includes facilities for reporting the exact version of *R* and associated packages used in an analysis so that, if needed, discrepancies between software versions can be tracked down and their importance evaluated. While users often think of *R* packages as providing new functionality, packages are also used to enhance reproducibility by encapsulating a single analysis. The package can contain data sets, vignette(s) describing the analysis, *R* functions that might have been written, scripts for key data processing stages, and documentation (via standard *R* help mechanisms) of what the functions, data, and packages are about.

The *Bioconductor* project adopts practices that facilitate reproducibility. Versions of *R* and *Bioconductor* are released twice each year. Each *Bioconductor* release is the result of development, in a separate branch, during the previous six months. The release is built daily against the corresponding version of *R* on Linux, Mac, and Windows platforms, with an extensive suite of tests performed. The `biocLite` function ensures that each release of *R* uses the corresponding *Bioconductor* packages. The user thus has access to stable and tested package versions. *R* and *Bioconductor* are effective tools for reproducible research.

R and *Bioconductor* exist on the leading portion of the software life cycle. Contributors are primarily from academic institutions, and are directly involved in novel research activities. New developments are made available in a familiar format, i.e., the *R* language, packaging, and build systems. The rich set of facilities in *R* (e.g., for advanced statistical analysis or visualization) and the extensive resources in *Bioconductor* (e.g., for annotation using third-party data such as Biomart or UCSC genome browser tracks) mean that innovations can be directly incorporated into existing work flows. The 'development' branches of *R* and *Bioconductor* provide an environment where contributors can explore new approaches without alienating their user base.

R and *Bioconductor* also fair well in terms of accessibility. The software is freely available. The source

Table 3.1: Selected *Bioconductor* packages for high-throughput sequence analysis.

Concept	Packages
Data representation	*IRanges*, *GenomicRanges*, *GenomicFeatures*, *Biostrings*, *BSgenome*, *girafe*.
Input / output	*ShortRead* (fastq), *Rsamtools* (bam), *rtracklayer* (gff, wig, bed), *VariantAnnotation* (vcf), *R453Plus1Toolbox* (454).
Annotation	*GenomicFeatures*, *ChIPpeakAnno*, *VariantAnnotation*.
Alignment	*gmapR*, *Rsubread*, *Biostrings*.
Visualization	*ggbio*, *Gviz*.
Quality assessment	*qrqc*, *seqbias*, *ReQON*, *htSeqTools*, *TEQC*, *Rolexa*, *ShortRead*.
RNA-seq	*BitSeq*, *cqn*, *cummeRbund*, *DESeq*, *DEXSeq*, *EDASeq*, *edgeR*, *gage*, *goseq*, *iASeq*, *tweeDEseq*.
ChIP-seq, etc.	*BayesPeak*, *baySeq*, *ChIPpeakAnno*, *chipseq*, *ChIPseqR*, *ChIPsim*, *CSAR*, *DiffBind*, *MEDIPS*, *mosaics*, *NarrowPeaks*, *nucleR*, *PICS*, *PING*, *REDseq*, *Repitools*, *TSSi*.
Variants	*VariantAnnotation*, *VariantTools*, *gmapR*
SNPs	*snpStats*, *GWASTools*, *hapFabia*, *GGtools*
Copy number	*cn.mops*, *genoset*, *CNAnorm*, *exomeCopy*, *seqmentSeq*.
Motifs	*MotifDb*, *BCRANK*, *cosmo*, *cosmoGUI*, *MotIV*, *seqLogo*, *rGADEM*.
3C, etc.	*HiTC*, *r3Cseq*.
Microbiome	*phyloseq*, *DirichletMultinomial*, *clstutils*, *manta*, *mcaGUI*.
Work flows	*QuasR*, *easyRNASeq*, *ArrayExpressHTS*, *Genominator*, *oneChannelGUI*, *rnaSeqMap*.
Database	*SRAdb*.

code is easily and fully accessible for critical evaluation. The *R* packaging and check system requires that all functions are documented. *Bioconductor* requires that each package contain vignettes to illustrate the use of the software. There are very active *R* and *Bioconductor* mailing lists for immediate support, and regular training and conference activities for professional development.

3.3 *Bioconductor* packages for high-throughput sequence analysis

Table 3.1 enumerates many of the packages available for sequence analysis. The table includes packages for representing sequence-related data (e.g., *GenomicRanges*, *Biostrings*), as well as domain-specific analysis such as RNA-seq (e.g., *edgeR*, *DEXSeq*), ChIP-seq (e.g,. *ChIPpeakAnno*, *DiffBind*), variants (e.g., *VariantAnnotation*, *VariantTools*, and SNPs and copy number variation (e.g., *genoset*, *ggtools*).

3.4 S4 Classes and methods

Bioconductor makes extensive use of 'S4' classes. Essential operations are sketched in Table 3.2. *Bioconductor* has tried to develop and use 'best practices' for use of S4 classes. Usually instances are created by a call to a *constructor*, such as `GRanges` (an object representing genomic ranges, with information on sequence, strand, start, and end coordinate of each range), or are returned by a function call that makes the object 'behind the scenes' (e.g., `readFastq`). Objects can have complicated structure, but users are not expected to have to concern themselves with the internal representation, just as the details of the S3 object returned by the `lm` function are not of direct concern. Instead, one might query the object to retrieve information; functions providing this functionality are sometimes called *accessors*, e.g., `seqnames`; the data that is returned by the accessor may involve some calculation, e.g., querying a data base, that the user can remain blissfully unaware of. It can be important to appreciate that objects can be related to one another, in particular inheriting

Table 3.2: Using S4 classes and methods.

Best practices	
`gr <- GRanges()`	'Constructor'; create an instance of the *GRanges* class
`seqnames(gr)`	'Accessor', extract information from an instance
`countOverlaps(gr1, gr2)`	A *method* implementing a *generic* with useful functionality
Older packages	
`s <- new("MutliSet)`	A constructor
`s@annotation`	A 'slot' accessor
Help	
`class(gr)`	Discover class of instance
`getClass(gr)`	Display class structure, e.g., inheritance
`showMethods(findOverlaps)`	Classes for which methods of `findOverlaps` are implemented
`showMethods(class="GRanges", where=search())`	Generics with methods implemented for the *GRanges* class, limited to currently loaded packages.
`class?GRanges`	Documentation for the *GRanges* class.
`method?"findOverlaps,GRanges,GRanges"`	Documentation for the `findOverlaps` method when the two arguments are both *GRanges* instances.
`selectMethod(findOverlaps, c("GRanges", "GRanges"))`	View source code for the method, including method 'dispatch'

parts of its internal structure and external behavior from other classes. For instance, the *GRanges* class inherits structure and behavior from the *GenomicRanges* class. The details of structural inheritance should not be important to the user, but the fact that once class inherits from another can be useful information to know.

One often calls a function in which one or more objects are arguments, e.g., `countOverlaps` can take two *GRanges* instances. The role of the function is to transform inputs into outputs. In the case of `countOverlaps` the transformation is to summarize the number of ranges in the second argument (the `subject` function argument) overlap with ranges in the first argument (the `query`) argument. This establishes a kind of contract, e.g., the return value of `countOverlaps` should be a non-negative integer vector, with as many elements as there are ranges in the `query` argument, and with a one-to-one correspondence between elements in the `query` input argument and the output. Having established such a contract, it can be convenient to write variations of `countOverlaps` that fulfill the contract but for different objects, e.g., when the arguments are instances of class *IRanges*, which do not have information about chromosomal sequence or strand. To indicate that the same contract is being fulfilled, and perhaps to simplify software development, one typically makes `countOverlaps` a *generic* function, and implements methods for different types of arguments.

Attending course and reading vignettes pages are obviously an excellent way to get an initial orientation about classes and methods that are available. It can be very helpful, as one becomes more proficient, to use the interactive help system to discover what can be done with the objects one has or the functions one knows about.

The `showMethods` function is a key entry point into discovery of available methods, e.g., `showMethods("countOverlaps")` to show methods defined on the `countOverlaps` generic, or `showMethods(class="GRanges", where=search())` to discover methods available to transform *GRanges* instances. The definition of a method can be retrieved as

```
> selectMethod(countOverlaps, c("GRanges", "GRanges"))
```

Exercise 4
Load the GenomicRanges package.

a. *Use* `getClass` *to discover the class structure of GRanges, paying particular attention to inheritance relationships summarized in the "Extends:" section of the display.*
b. *Use* `showMethods` *to see what methods are defined for the* `countOverlaps` *function.*
c. *There are many methods defined for* `countOverlaps`*, but none are listed for the GRanges,GRanges combination of arguments. Yet* `countOverlaps` does *work when provided with two GRanges arguments. Why is that?*

Solution: Here we load the package and ask about class structure.

```
> library(GenomicRanges)
> getClass("GRanges")

Class "GRanges" [package "GenomicRanges"]

Slots:

Name:         seqnames          ranges          strand elementMetadata
Class:             Rle         IRanges             Rle       DataFrame

Name:          seqinfo        metadata
Class:         Seqinfo            list

Extends:
Class "GenomicRanges", directly
Class "Vector", by class "GenomicRanges", distance 2
Class "GenomicRangesORmissing", by class "GenomicRanges", distance 2
Class "GenomicRangesORGRangesList", by class "GenomicRanges", distance 2
Class "Annotated", by class "GenomicRanges", distance 3
```

GRanges extends several classes, including *GenomicRanges*. Method defined on the `countOverlaps` generic can be discovered with

```
> showMethods("countOverlaps")

Function: countOverlaps (package IRanges)
query="ANY", subject="Vector"
query="ANY", subject="missing"
query="GAlignmentsList", subject="GAlignmentsList"
query="GAlignmentsList", subject="Vector"
query="GRangesList", subject="GRangesList"
query="GRangesList", subject="Vector"
query="GappedAlignmentPairs", subject="Vector"
query="GappedAlignments", subject="GappedAlignments"
query="GappedAlignments", subject="Vector"
query="GenomicRanges", subject="GenomicRanges"
query="GenomicRanges", subject="Vector"
query="RangedData", subject="RangedData"
query="RangedData", subject="RangesList"
query="RangesList", subject="RangedData"
query="RangesList", subject="RangesList"
query="SummarizedExperiment", subject="SummarizedExperiment"
```

```
query="SummarizedExperiment", subject="Vector"
query="Vector", subject="GAlignmentsList"
query="Vector", subject="GRangesList"
query="Vector", subject="GappedAlignmentPairs"
query="Vector", subject="GappedAlignments"
query="Vector", subject="GenomicRanges"
query="Vector", subject="SummarizedExperiment"
query="Vector", subject="ViewsList"
query="ViewsList", subject="Vector"
query="ViewsList", subject="ViewsList"
```

(quotation marks are, in this case, optional). Note that there is no method defined for the *GRanges,GRanges* combination of arguments. Yet `countOverlaps` *does* work. . . (skim over the details of the se objects; we are using a constructor to make genomic ranges on plus strand of chromosome 1; there are two ranges in `gr0`, and one in `gr1`.

```
> gr0 <- GRanges("chr1", IRanges(start=c(10, 20), width = 5), "+")
> gr1 <- GRanges("chr1", IRanges(start=12, end=18), "+")
> countOverlaps(gr0, gr1)
```

```
[1] 1 0
```

`gr1` overlaps the first range of `gr0`, but not the second, and we end up with a vector of counts `c(1, 0)`. The reason that this 'works' is because of inheritance – *GRanges* extends *GenomicRanges*, and we end up selecting the inherited method *countOverlaps,GenomicRanges,GenomicRanges-method*.

3.5 Help!

S4 classes, generics, and methods To illustrate how help work with S4 classes and generics, consider the *DNAStringSet* class `complement` generic in the *Biostrings* package:

```
> library(Biostrings)
> showMethods(complement)
```

```
Function: complement (package Biostrings)
x="DNAString"
x="DNAStringSet"
x="MaskedDNAString"
x="MaskedRNAString"
x="RNAString"
x="RNAStringSet"
x="XStringViews"
```

(Most) methods defined on the `DNAStringSet` class of *Biostrings* and available on the current search path can be found with

```
> showMethods(class="DNAStringSet", where=search())
```

Obtaining help on S4 classes and methods requires syntax such as

```
> class ? DNAStringSet
> method ? "complement,DNAStringSet"
```

The specification of method and class in the latter must not contain a space after the comma.

Chapter 4

Sequencing

Recent technological developments introduce high-throughput sequencing approaches. A variety of experimental protocols and analysis work flows address gene expression, regulation, and encoding of genetic variants. Experimental protocols produce a large number (tens of millions per sample) of short (e.g., 35-150, single or paired-end) nucleotide sequences. These are aligned to a reference or other genome. Analysis work flows use the alignments to infer levels of gene expression (RNA-seq), binding of regulatory elements to genomic locations (ChIP-seq), or prevalence of structural variants (e.g., SNPs, short indels, large-scale genomic rearrangements). Sample sizes range from minimal replication (e.g,. 2 samples per treatment group) to thousands of individuals.

4.1 Technologies

The most common 'second generation' technologies readily available to labs are

- Illumina single- and paired-end reads. Short ($\approx$ 100 per end) and very numerous. Flow cell, lane, bar-code.
- Roche 454. 100's of nucleotides, 100,000's of reads.
- Life Technologies SOLiD. Unique 'color space' model.
- Complete Genomics. Whole genome sequence / variants / etc as a service; end user gets derived results.

Figure 4.1 illustrates Illumina and 454 sequencing. *Bioconductor* has good support for Illumina and Roche 454 sequencing products, and for derived data such as aligned reads or called variants; use of SOLiD color space reads typically requires conversion to FASTQ files that undermine the benefit of the color space model.

All second-generation technologies rely on PCR and other techniques to generate reads from samples that represent aggregations of many DNA molecules. 'Third-generation' technologies shift to single-molecule sequencing, with relevant players including Pacific Biosciences and IonTorent. This data is not widely available, and will not be discussed further.

The most common data in *Bioconductor* work flows is from Illumina sequencers. Reads are either single-end or paired-end. Single-end reads represent $30- \approx 100$ nucleotides sequenced from DNA that has been sheared into ≈ 300 nucleotide fragments. Paired-end reads represent $30- \approx 100$ nucleotide reads that are paired, and from both ends of the ≈ 300 fragment.

Sequence data can be derived from a tremendous diversity of experiments. Some of the most common include:

RNA-seq Sequencing of reverse-complemented mRNA from the entire expressed transcriptome, typically. Used for *differential expression* studies like micro-arrays, or for *novel transcript discovery*.

DNA-seq Sequencing of whole or targeted (e.g., exome) genomic DNA. Common goals include *SNP* detection, *indel* and other structural polymorphisms, and *CNV* (copy number variation). DNA-seq is also used for *de novo* assembly, but *de novo* assembly is not an area where *Bioconductor* contributes.

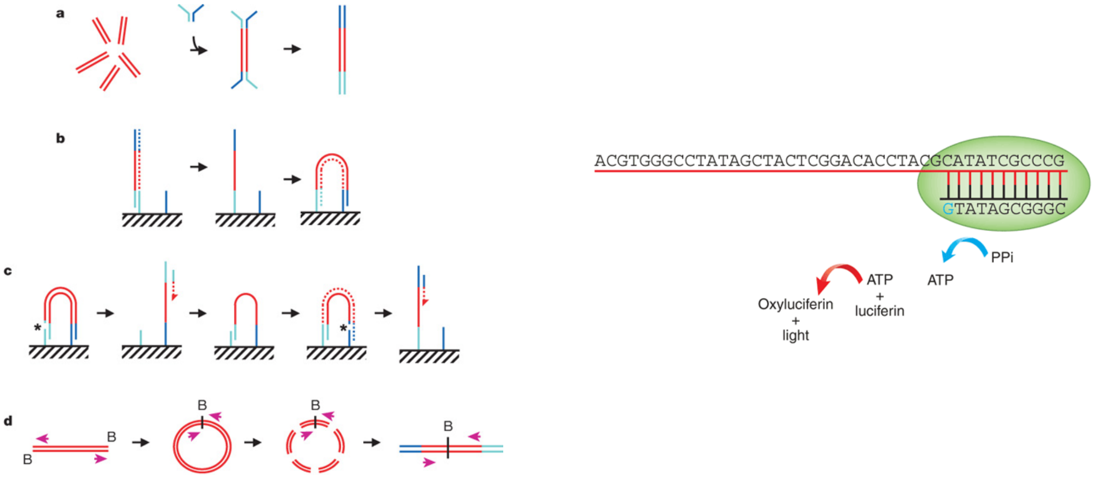


Figure 4.1: High-throughput sequencing. Left: Illumina bridge PCR [2]; mis-call errors. Right: Roche 454 [24]; homopolymer errors.

ChIP-seq ChIP (chromatin immuno-precipitation) is used to enrich genomic DNA for regulatory elements, followed by sequencing and mapping of the enriched DNA to a reference genome. The initial statistical challenge is to identify regions where the mapped reads are enriched relative to a sample that did not undergo ChIP[20]; a subsequent task is to identify differential binding across a designed experiment, e.g., [23].

Metagenomics Sequencing generates sequences from samples containing multiple species, typically microbial communities sampled from niches such as the human oral cavity. Goals include inference of *species composition* (when sequencing typically targets phylogenetically informative genes such as 16S) or *metabolic contribution*.

4.2 Data

4.2.1 A running example: the *pasilla* data set

As a running example, we use the *pasilla* data set, derived from [3]. The authors investigate conservation of RNA regulation between *D. melanogaster* and mammals. Part of their study used RNAi and RNA-seq to identify exons regulated by Pasilla (*ps*), the *D. melanogaster* ortholog of mammalian NOVA1 and NOVA2. Briefly, their experiment compared gene expression as measured by RNAseq in S2-DRSC cells cultured with, or without, a 444bp dsRNA fragment corresponding to the *ps* mRNA sequence. Their assessment investigated differential exon use, but our worked example will focus on gene-level differences. For several examples we look at a subset of the *ps* data, corresponding to reads obtained from lanes of their RNA-seq experiment, and to the same reads aligned to a *D. melanogaster* reference genome. Reads were obtained from GEO and the Short Read Archive (SRA), and were aligned to the *D. melanogaster* reference genome *dm3* as described in the *pasilla* experiment data package.

4.2.2 Work flows

At a very high level, one can envision a work flow that starts with a challenging biological question (how does *ps* influence gene and transcript regulation?). The biological question is framed in terms of wet-lab protocols coupled with an appropriate and all-important experimental design. There are several well-known statistical at this stage, common to any experimental data What treatments are going to be applied? How many replicates will there be of each? Is there likely to be sufficient power to answer the biologically relevant

Table 4.1: Common file types (e.g., `http://genome.ucsc.edu/FAQ/FAQformat.html`) and *Bioconductor* packages used for input.

File	Description	Package
FASTQ	Unaligned sequences: identifier, sequence, and encoded quality score tuples	*ShortRead*
BAM	Aligned sequences: identifier, sequence, reference sequence name, strand position, cigar and additional tags	*Rsamtools*
GFF, GTF	Gene annotations: reference sequence name, data source, feature type, start and end positions, strand, etc.	*rtracklayer*
BED	Range-based annotation: reference sequence name, start, end coordinates.	*rtracklayer*
WIG, bigWig	'Continuous' single-nucleotide annotation.	*rtracklayer*
VCF	Called single nucleotide, indel, copy number, and structural variants, often compressed and indexed (with *Rsamtools* `bgzip`, `indexTabix`)	*VariantAnnotation*

question? Reality is also important at this stage, as evidenced in the *pasilla* data where, as we will see, samples were collected using different methods (single versus paired end reads) over a time when there were rapid technological changes. Such reality often introduces confounding factors that require appropriate statistical treatment in subsequent analysis.

The work flow proceeds with data generation, involving both a wet-lab (sample preparation) component and actual sequencing. It is essential to acknowledge the biases and artifacts that are introduced at each of these stages. Sample preparation involves non-trivial amounts of time and effort. Large studies are likely to have batch effects (e.g., because work was done by different lab members, or different batches of reagent). Samples might have been prepared in ways that are likely to influence down-stream analysis, e.g., using a protocol involving PCR and hence introducing opportunities for sample-specific bias. DNA isolation protocols may introduce many artifacts, e.g., non-uniform representation of reads across the length of expressed genes in RNA-seq. The sequencing reaction itself is far from bias-free, with known artifacts of called base frequency, cycle-dependent accuracy and bias, non-uniform coverage, etc. At a minimum, the research needs to be aware of the opportunities for bias that can be introduced during sample preparation and sequencing.

The informatics component of work flows becomes increasing important during and after sequence generation. The sequencer is often treated as a 'black box', producing short reads consisting of 10's to 100's of nucleotides and with associated quality scores. Usually, the chemistry and informatics processing pipeline are sufficiently well documented that one can arrive at an understanding of biases and quality issues that might be involved; such an understanding is likely to be particularly important when embarking on questions or using protocols that are at the fringe of standard practice (where, after all, the excitement is).

The first real data seen by users are *fastq* files (Table 4.1). These files are often simple text files consisting of many millions of records, and are described in greater detail in Section 5.2. The center performing the sequencing typically vets results for quality, but these quality measures are really about the performance of their machines. It is very important to assess quality with respect to the experiment being undertaken – Are the numbers of reads consistent across samples? Is the GC content and other observable aspects of the reads consistent with expectation? Are there anomalies in the sequence results that reflect primers or other reagents used during sample preparation? Are well-known artifacts of the protocol used evident in the reads in hand?

The next step in many work flows involves alignment of reads to a reference genome. There are many aligners available, including BWA [13], Bowtie / Bowtie2 [12], and GSNAP; merits of these are discussed in the literature. *Bioconductor* packages 'wrapping' these tools are increasingly common (e.g., *Rbowtie*, *gmapR*; *cummeRbund* for parsing output of the *cufflinks* transcript discovery pathway). There are also alignment algorithms implemented in *Bioconductor* (e.g., `matchPDict` in the *Biostrings* package, and the

Rsubread package); `matchPDict` is particularly useful for flexible alignment of moderately sized subsets of data. Most main-stream aligners produce output in 'SAM' or 'BAM' (binary alignment) format. BAM files are the primary starting point for many analyses, and their manipulation and use in *Bioconductor* is introduced in Section 6.2.

Common analyses often use well-established third-party tools for initial stages of the analysis; some of these have *Bioconductor* counterparts that are particularly useful when the question under investigation does not meet the assumptions of other facilities. Some common work flows (a more comprehensive list is available on the SeqAnswers wiki[1]) include:

ChIP-seq ChIP-seq experiments typically use DNA sequencing to identify regions of genomic DNA enriched in prepared samples relative to controls. A central task is thus to identify peaks, with common tools including *MACS* and *PeakRanger*.

RNA-seq In addition to the aligners mentioned above, RNA-seq for differential expression might use the *HTSeq*[2] python tools for counting reads within regions of interest (e.g., known genes) or a pipeline such as the *bowtie* (basic alignment) / *tophat* (splice junction mapper) / *cufflinks* (esimated isoform abundance) (e.g., [3]) or *RSEM*[4] suite of tools for estimating transcript abundance.

DNA-seq especially variant calling can be facilitated by software such as the GATK[5] toolkit.

There are many *R* packages that replace or augment the analyses outlined above, as summarized in Table 3.1.

Programs such as those outlined the previous paragraph often rely on information about gene or other structure as input, or produce information about chromosomal locations of interesting features. The GTF and BED file formats are common representations of this information. Representing these files as *R* data structures is often facilitated by the *rtracklayer* package. We explore these files in Chapter 14. Variants are very commonly represented in VCF (Variant Call Format) files; these are explored in Chapter 12.

[1] http://seqanswers.com/wiki/RNA-Seq

[2] http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html

[3] http://bowtie-bio.sourceforge.net/index.shtml

[4] http://deweylab.biostat.wisc.edu/rsem/

[5] http://www.broadinstitute.org/gatk/

Chapter 5

Strings and Reads

5.1 DNA (and other) Strings with the *Biostrings* package

The *Biostrings* package provides tools for working with sequences. The essential data structures are *DNAString* and *DNAStringSet*, for working with one or multiple DNA sequences. The *Biostrings* package contains additional classes for representing amino acid and general biological strings. The *BSgenome* and related packages (e.g., *BSgenome.Dmelanogaster.UCSC.dm3*) are used to represent whole-genome sequences. Table 5.2 summarizes common operations; The following exercise explores these packages.

Exercise 5
The objective of this exercise is to calculate the GC content of the exons of a single gene. We jump into the middle of some of the data structures common in Bioconductor; these are introduced more thoroughly in later exercises..

Load the BSgenome.Dmelanogaster.UCSC.dm3 *data package, containing the UCSC representation of* D. melanogaster *genome assembly dm3. Discover the content of the package by evaluating* `Dmelanogaster`*.*

Load the SequenceAnalysisData package, and evaluate the command `data(ex)` *to load an example of a GRangesList object. the GRangesList represents coordinates of exons in the* D. melanogaster *genome, grouped by gene.*

Look at `ex[1]`*. These are the genomic coordinates of the first gene in the* `ex` *object. Load the* D. melanogaster *chromosome that this gene is on by subsetting the* `Dmelanogaster` *object.*

Use `Views` *to create views on to the chromosome that span the start and end coordinates of all exons in the first gene; the start and end coordinates are accessed with* `start(ex[[1]])` *and similar.*

Develop a function `gcFunction` *to calculate GC content. Use this to calculate the GC content in each of the exons.*

Solution: Here we load the *D. melanogaster* genome, select a single chromosome, and create `Views` that reflect the ranges of the `FBgn0002183`.

Table 5.1: Selected *Bioconductor* packages for representing strings and reads.

Package	Description
Biostrings	Classes (e.g., *DNAStringSet*) and methods (e.g., `alphabetFrequency`, `pairwiseAlignment`) for representing and manipulating DNA and other biological sequences.
BSgenome	Representation and manipulation of large (e.g., whole-genome) sequences.
ShortRead	I/O and manipulation of FASTQ files.

Table 5.2: Operations on strings in the *Biostrings* package.

	Function	Description
Access	`length`, `names`	Number and names of sequences
	`[`, `head`, `tail`, `rev`	Subset, first, last, or reverse sequences
	`c`	Concatenate two or more objects
	`width`, `nchar`	Number of letters of each sequence
	`Views`	Light-weight sub-sequences of a sequence
Compare	`==`, `!=`, `match`, `%in%`	Element-wise comparison
	`duplicated`, `unique`	Analog to `duplicated` and `unique` on character vectors
	`sort`, `order`	Locale-independent sort, order
	`split`, `relist`	Split or relist objects to, e.g., *DNAStringSetList*
Edit	`subseq`, `subseq<-`	Extract or replace sub-sequences in a set of sequences
	`reverse`, `complement`	Reverse, complement, or reverse-complement DNA
	`reverseComplement`	
	`translate`	Translate DNA to Amino Acid sequences
	`chartr`	Translate between letters
	`replaceLetterAt`	Replace letters at a set of positions by new letters
	`trimLRPatterns`	Trim or find flanking patterns
Count	`alphabetFrequency`	Tabulate letter occurrence
	`letterFrequency`	
	`letterFrequencyInSlidingView`	
	`consensusMatrix`	Nucleotide × position summary of letter counts
	`dinucleotideFrequency`	2-mer, 3-mer, and k-mer counting
	`trinucleotideFrequency`	
	`oligonucleotideFrequency`	
	`nucleotideFrequencyAt`	Nucleotide counts at fixed sequence positions
Match	`matchPattern`, `countPattern`	Short patterns in one or many (`v*`) sequences
	`vmatchPattern`, `vcountPattern`	
	`matchPDict`, `countPDict`	Short patterns in one or many (`v*`) sequences (mismatch only)
	`whichPDict`, `vcountPDict`	
	`vwhichPDict`	
	`pairwiseAlignment`	Needleman-Wunsch, Smith-Waterman, etc. pairwise alignment
	`matchPWM`, `countPWM`	Occurrences of a position weight matrix
	`matchProbePair`	Find left or right flanking patterns
	`findPalindromes`	Palindromic regions in a sequence. Also `findComplementedPalindromes`
	`stringDist`	Levenshtein, Hamming, or pairwise alignment scores
I/0	`readDNAStringSet`	FASTA (or sequence only from FASTQ). Also `readBStringSet`, `readRNAStringSet`, `readAAStringSet`
	`writeXStringSet`	
	`writePairwiseAlignments`	Write `pairwiseAlignment` as "pair" format
	`readDNAMultipleAlignment`	Multiple alignments (FASTA, "stockholm", or "clustal"). Also `readRNAMultipleAlignment`, `readAAMultipleAlignment`
	`write.phylip`	

```
> library(BSgenome.Dmelanogaster.UCSC.dm3)
> Dmelanogaster

Fly genome
|
| organism: Drosophila melanogaster (Fly)
| provider: UCSC
| provider version: dm3
| release date: Apr. 2006
| release name: BDGP Release 5
|
| single sequences (see '?seqnames'):
|   chr2L      chr2R      chr3L      chr3R      chr4       chrX       chrU
|   chrM       chr2LHet   chr2RHet   chr3LHet   chr3RHet   chrXHet    chrYHet
|   chrUextra
|
| multiple sequences (see '?mseqnames'):
|   upstream1000  upstream2000  upstream5000
|
| (use the '$' or '[[' operator to access a given sequence)

> library(SequenceAnalysisData)
> data(ex)
> ex[1]

GRangesList of length 1:
$FBgn0002183
GRanges with 9 ranges and 0 metadata columns:
      seqnames             ranges strand
         <Rle>          <IRanges>  <Rle>
  [1]    chr3L [1871574, 1871917]      -
  [2]    chr3L [1872354, 1872470]      -
  [3]    chr3L [1872582, 1872735]      -
  [4]    chr3L [1872800, 1873062]      -
  [5]    chr3L [1873117, 1873983]      -
  [6]    chr3L [1874041, 1875218]      -
  [7]    chr3L [1875287, 1875586]      -
  [8]    chr3L [1875652, 1875915]      -
  [9]    chr3L [1876110, 1876336]      -

---
seqlengths:
     chr2L  chr2LHet     chr2R  chr2RHet ...   chrXHet   chrYHet      chrM
  23011544    368872  21146708   3288761 ...    204112    347038     19517

> nm <- "chr3L"
> chr <- Dmelanogaster[[nm]]
> v <- Views(chr, start=start(ex[[1]]), end=end(ex[[1]]))
```

Here is the `gcFunction` helper function to calculate GC content:

```
> gcFunction <-
+     function(x)
+ {
+     alf <- alphabetFrequency(x, as.prob=TRUE)
+     rowSums(alf[,c("G", "C")])
+ }
```

The `gcFunction` is really straight-forward: it invokes the function `alphabetFrequency` from the *Biostrings* package. This returns a simple matrix of exon × nucleotide probabilities. The row sums of the `G` and `C` columns of this matrix are the GC contents of each exon.

The subject GC content is

```
> subjectGC <- gcFunction(v)
```

5.2 Reads and the *ShortRead* package

Short read formats The Illumina GAII and HiSeq technologies generate sequences by measuring incorporation of florescent nucleotides over successive PCR cycles. These sequencers produce output in a variety of formats, but *FASTQ* is ubiquitous. Each read is represented by a record of four components:

```
@SRR031724.1 HWI-EAS299_4_30M2BAAXX:5:1:1513:1024 length=37
GTTTTGTCCAAGTTCTGGTAGCTGAATCCTGGGGCGC
+SRR031724.1 HWI-EAS299_4_30M2BAAXX:5:1:1513:1024 length=37
IIIIIIIIIIIIIIIIIIIIIIIIIIII+HIIII<IE
```

The first and third lines (beginning with `@` and `+` respectively) are unique identifiers. The identifier produced by the sequencer typically includes a machine id followed by colon-separated information on the lane, tile, x, and y coordinate of the read. The example illustrated here also includes the SRA accession number, added when the data was submitted to the archive. The machine identifier could potentially be used to extract information about batch effects. The spatial coordinates (lane, tile, x, y) are often used to identify optical duplicates; spatial coordinates can also be used during quality assessment to identify artifacts of sequencing, e.g., uneven amplification across the flow cell, though these spatial effects are rarely pursued.

The second and fourth lines of the FASTQ record are the nucleotides and qualities of each cycle in the read. This information is given in 5' to 3' orientation as seen by the sequencer. A letter `N` in the sequence is used to signify bases that the sequencer was not able to call. The fourth line of the FASTQ record encodes the quality (confidence) of the corresponding base call. The quality score is encoded following one of several conventions, with the general notion being that letters later in the visible ASCII alphabet

```
!"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJKLMNO
PQRSTUVWXYZ[\]^_`abcdefghijklmnopqrstuvwxyz{|}~
```

are of higher quality; this is developed further below. Both the sequence and quality scores may span multiple lines.

Technologies other than Illumina use different formats to represent sequences. Roche 454 sequence data is generated by 'flowing' labeled nucleotides over samples, with greater intensity corresponding to longer runs of A, C, G, or T. This data is represented as a series of 'flow grams' (a kind of run-length encoding of the read) in Standard Flowgram Format (SFF). The *Bioconductor* package *R453Plus1Toolbox* has facilities for parsing SFF files, but after quality control steps the data are frequently represented (with some loss of information) as FASTQ. SOLiD technologies produce sequence data using a 'color space' model. This data is not easily read in to *R*, and much of the error-correcting benefit of the color space model is lost when converted to FASTQ; SOLiD sequences are not well-handled by *Bioconductor* packages.

Short reads in *R* FASTQ files can be read in to *R* using the `readFastq` function from the *ShortRead* package. Use this function by providing the path to a FASTQ file. There are sample data files available in the *SequenceAnalysisData* package, each consisting of 1 million reads from a lane of the Pasilla data set.

```
> library(ShortRead)
> bigdata <- system.file("bigdata", package="SequenceAnalysisData")
> fastqDir <- file.path(bigdata, "fastq")
> fastqFiles <- dir(fastqDir, full=TRUE)
> fq <- readFastq(fastqFiles[1])
> fq
```

```
class: ShortReadQ
length: 1000000 reads; width: 37 cycles
```

The data are represented as an object of class *ShortReadQ*.

```
> head(sread(fq), 3)
```

```
  A DNAStringSet instance of length 3
    width seq
[1]    37 GTTTTGTCCAAGTTCTGGTAGCTGAATCCTGGGGCGC
[2]    37 GTTGTCGCATTCCTTACTCTCATTCGGGAATTCTGTT
[3]    37 GAATTTTTTGAGAGCGAAATGATAGCCGATGCCCTGA
```

```
> head(quality(fq), 3)
```

```
class: FastqQuality
quality:
  A BStringSet instance of length 3
    width seq
[1]    37 IIIIIIIIIIIIIIIIIIIIIIIIIIII+HIIII<IE
[2]    37 IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
[3]    37 IIIIIIIIIIIIIIIIIIIIII'IIIIIGBIIII2I+
```

```
> head(id(fq), 3)
```

```
  A BStringSet instance of length 3
    width seq
[1]    58 SRR031724.1 HWI-EAS299_4_30M2BAAXX:5:1:1513:1024 length=37
[2]    57 SRR031724.2 HWI-EAS299_4_30M2BAAXX:5:1:937:1157 length=37
[3]    58 SRR031724.4 HWI-EAS299_4_30M2BAAXX:5:1:1443:1122 length=37
```

The *ShortReadQ* class illustrates *class inheritance*. It extends the *ShortRead* class

```
> getClass("ShortReadQ")
```

```
Class "ShortReadQ" [package "ShortRead"]

Slots:

Name:       quality        sread           id
Class: QualityScore DNAStringSet   BStringSet

Extends:
Class "ShortRead", directly
Class ".ShortReadBase", by class "ShortRead", distance 2

Known Subclasses: "AlignedRead"
```

Methods defined on *ShortRead* are available for *ShortReadQ*.

```
> showMethods(class="ShortRead", where="package:ShortRead")
```

For instance, the `width` can be used to demonstrate that all reads consist of 37 nucleotides.

```
> table(width(fq))

     37
1000000
```

The `alphabetByCycle` function summarizes use of nucleotides at each cycle in a (equal width) *ShortReadQ* or *DNAStringSet* instance.

```
> abc <- alphabetByCycle(sread(fq))
> abc[1:4, 1:8]

        cycle
alphabet   [,1]   [,2]   [,3]   [,4]   [,5]   [,6]   [,7]   [,8]
       A  78194 153156 200468 230120 283083 322913 162766 220205
       C 439302 265338 362839 251434 203787 220855 253245 287010
       G 397671 270342 258739 356003 301640 247090 227811 246684
       T  84833 311164 177954 162443 211490 209142 356178 246101
```

FASTQ files are getting larger. A very common reason for looking at data at this early stage in the processing pipeline is to explore sequence quality. In these circumstances it is often not necessary to parse the entire FASTQ file. Instead create a representative sample

```
> sampler <- FastqSampler(fastqFiles[1], 1000000)
> yield(sampler) # sample of 1000000 reads

class: ShortReadQ
length: 1000000 reads; width: 37 cycles
```

A second common scenario is to pre-process reads, e.g., trimming low-quality tails, adapter sequences, or artifacts of sample preparation. The *FastqStreamer* class can be used to 'stream' over the fastq files in chunks, processing each chunk independently.

ShortRead contains facilities for quality assessment of FASTQ files. Here we generate a report from a sample of 1 million reads from each of our files and display it in a web browser

```
> qas0 <- Map(function(fl, nm) {
+     fq <- FastqSampler(fl)
+     qa(yield(fq), nm)
+ }, fastqFiles,
+    sub("_subset.fastq", "", basename(fastqFiles)))
> qas <- do.call(rbind, qas0)
> rpt <- report(qas, dest=tempfile())
> browseURL(rpt)
```

A report from a larger subset of the experiment is available

```
> rpt <- system.file("GSM461176_81_qa_report", "index.html",
+                    package="SequenceAnalysisData")
> browseURL(rpt)
```

Exercise 6

Use the file path `bigdata` *and the* `file.path` *and* `dir` *functions to locate the fastq file from [3] (the file was obtained as described in the pasilla experiment data package).*

Input the fastq files using `readFastq` *from the* ShortRead *package.*

Use `alphabetFrequency` *to summarize the GC content of all reads (hint: use the* `sread` *accessor to extract the reads, and the* `collapse=TRUE` *argument to the* `alphabetFrequency` *function). Using the helper function* `gcFunction` *defined elsewhere in this document, draw a histogram of the distribution of GC frequencies across reads.*

Use `alphabetByCycle` *to summarize the frequency of each nucleotide, at each cycle. Plot the results using* `matplot`*, from the graphics package.*

As an advanced exercise, and if on Mac or Linux, use the parallel package and `mclapply` *to read and summarize the GC content of reads in two files in parallel.*

Solution: Discovery:

```
> dir(bigdata)
```

```
[1] "bam"   "fastq"
```

```
> fls <- dir(file.path(bigdata, "fastq"), full=TRUE)
```

Input:

```
> fq <- readFastq(fls[1])
```

GC content:

```
> alf0 <- alphabetFrequency(sread(fq), as.prob=TRUE, collapse=TRUE)
> sum(alf0[c("G", "C")])
```

```
[1] 0.5457237
```

A histogram of the GC content of individual reads is obtained with

```
> gc <- gcFunction(sread(fq))
> hist(gc)
```

Alphabet by cycle:

```
> abc <- alphabetByCycle(sread(fq))
> matplot(t(abc[c("A", "C", "G", "T"),]), type="l")
```

Advanced (Mac, Linux only): processing on multiple cores.

```
> library(parallel)
> gc0 <- mclapply(fls, function(fl) {
+   fq <- readFastq(fl)
+   gc <- gcFunction(sread(fq))
+   table(cut(gc, seq(0, 1, .05)))
+ })
> ## simplify list of length 2 to 2-D array
> gc <- simplify2array(gc0)
> matplot(gc, type="s")
```

Exercise 7
Use `quality` *to extract the quality scores of the short reads. Interpret the encoding qualitatively.*

Convert the quality scores to a numeric matrix, using `as`*. Inspect the numeric matrix (e.g., using* `dim`*) and understand what it represents.*

Use `colMeans` *to summarize the average quality score by cycle. Use* `plot` *to visualize this.*

Solution:

```
> head(quality(fq))

class: FastqQuality
quality:
  A BStringSet instance of length 6
    width seq
[1]    37 IIIIIIIIIIIIIIIIIIIIIIIIIIII+HIIII<IE
[2]    37 IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII
[3]    37 IIIIIIIIIIIIIIIIIIIIII'IIIIIGBIIII2I+
[4]    37 IIIIIIIIIIIIIIIIIIIIIIII,II*E,&4HI++B
[5]    37 IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII&.$
[6]    37 III.IIIIIIIIIIIIIIIIIII%IIE(-EIH<IIII

> qual <- as(quality(fq), "matrix")
> dim(qual)

[1] 1000000      37

> plot(colMeans(qual), type="b")
```

Exercise 8
As an independent exercise, visit the qrqc landing page and explore the package vignette. Use the qrqc package (you may need to install this) to generate base and average quality plots for the data, like those in the report generated by ShortRead.

Chapter 6

Ranges and Alignments

Ranges describe both features of interest (e.g., genes, exons, promoters) and reads aligned to the genome. *Bioconductor* has very powerful facilities for working with ranges, some of which are summarized in Table 6.1.

6.1 Ranges and the *GenomicRanges* package

Next-generation sequencing data consists of a large number of short reads. These are, typically, aligned to a reference genome. Basic operations are performed on the alignment, asking e.g., how many reads are aligned in a genomic range defined by nucleotide coordinates (e.g., in the exons of a gene), or how many nucleotides from all the aligned reads cover a set of genomic coordinates. How is this type of data, the aligned reads and the reference genome, to be represented in *R* in a way that allows for effective computation?

The *IRanges*, *GenomicRanges*, and *GenomicFeatures* *Bioconductor* packages provide the essential infrastructure for these operations; we start with the *GRanges* class, defined in *GenomicRanges*.

GRanges Instances of *GRanges* are used to specify genomic coordinates. Suppose we wish to represent two *D. melanogaster* genes. The first is located on the positive strand of chromosome 3R, from position 19967117 to 19973212. The second is on the minus strand of the X chromosome, with 'left-most' base at 18962306, and right-most base at 18962925. The coordinates are *1-based* (i.e., the first nucleotide on a chromosome is numbered 1, rather than 0), *left-most* (i.e., reads on the minus strand are defined to 'start' at the left-most coordinate, rather than the 5' coordinate), and *closed* (the start and end coordinates are included in the range; a range with identical start and end coordinates has width 1, a 0-width range is represented by the special construct where the end coordinate is one less than the start coordinate).

A complete definition of these genes as *GRanges* is:

Table 6.1: Selected *Bioconductor* packages for representing and manipulating ranges, strings, and other data structures.

Package	Description
IRanges	Defines important classes (e.g., *IRanges*, *Rle*) and methods (e.g., `findOverlaps`, `countOverlaps`) for representing and manipulating ranges of consecutive values. Also introduces *DataFrame*, *SimpleList* and other classes tailored to representing very large data.
GenomicRanges	Range-based classes tailored to sequence representation (e.g., *GRanges*, *GRangesList*), with information about strand and sequence name.
GenomicFeatures	Foundation for manipulating data bases of genomic ranges, e.g., representing coordinates and organization of exons and transcripts of known genes.

```
> genes <- GRanges(seqnames=c("3R", "X"),
+                  ranges=IRanges(
+                      start=c(19967117, 18962306),
+                      end=c(19973212, 18962925)),
+                  strand=c("+", "-"),
+                  seqlengths=c(`3R`=27905053L, `X`=22422827L))
```

The components of a *GRanges* object are defined as vectors, e.g., of `seqnames`, much as one would define a *data.frame*. The start and end coordinates are grouped into an *IRanges* instance. The optional `seqlengths` argument specifies the maximum size of each sequence, in this case the lengths of chromosomes 3R and X in the 'dm2' build of the *D. melanogaster* genome. This data is displayed as

```
> genes
```

```
GRanges with 2 ranges and 0 metadata columns:
      seqnames               ranges strand
         <Rle>            <IRanges>  <Rle>
  [1]       3R [19967117, 19973212]      +
  [2]        X [18962306, 18962925]      -
  ---
  seqlengths:
         3R        X
   27905053 22422827
```

For the curious, the gene coordinates and sequence lengths are derived from the *org.Dm.eg.db* package for genes with Flybase identifiers FBgn0039155 and FBgn0085359, using the annotation facilities described in Chapter 13.

The *GRanges* class has many useful methods defined on it. Consult the help page

```
> ?GRanges
```

and package vignettes (especially 'An Introduction to *GenomicRanges*')

```
> vignette(package="GenomicRanges")
```

for a comprehensive introduction. A *GRanges* instance can be subset, with accessors for getting and updating information.

```
> genes[2]
```

```
GRanges with 1 range and 0 metadata columns:
      seqnames               ranges strand
         <Rle>            <IRanges>  <Rle>
  [1]        X [18962306, 18962925]      -
  ---
  seqlengths:
         3R        X
   27905053 22422827
```

```
> strand(genes)
```

```
factor-Rle of length 2 with 2 runs
  Lengths: 1 1
  Values : + -
Levels(3): + - *
```

```
> width(genes)

[1] 6096  620

> length(genes)

[1] 2

> names(genes) <- c("FBgn0039155", "FBgn0085359")
> genes  # now with names

GRanges with 2 ranges and 0 metadata columns:
              seqnames               ranges strand
                 <Rle>            <IRanges>  <Rle>
  FBgn0039155       3R [19967117, 19973212]      +
  FBgn0085359        X [18962306, 18962925]      -
  ---
  seqlengths:
         3R        X
   27905053 22422827
```

`strand` returns the strand information in a compact representation called a *run-length encoding*, this is introduced in greater detail below. The 'names' could have been specified when the instance was constructed; once named, the *GRanges* instance can be subset by name like a regular vector.

As the `GRanges` function suggests, the *GRanges* class extends the *IRanges* class by adding information about `seqnames`, `strand`, and other information particularly relevant to representing ranges that are on genomes. The *IRanges* class and related data structures (e.g., *RangedData*) are meant as a more general description of ranges defined in an arbitrary space. Many methods implemented on the *GRanges* class are 'aware' of the consequences of genomic location, for instance treating ranges on the minus strand differently (reflecting the 5' orientation imposed by DNA) from ranges on the plus strand.

Operations on ranges The *GRanges* class has many useful methods from the *IRanges* class; some of these methods are illustrated here. We use *IRanges* to illustrate these operations to avoid complexities associated with strand and seqnames, but the operations are comparable on *GRanges*. We begin with a simple set of ranges:

```
> ir <- IRanges(start=c(7, 9, 12, 14, 22:24),
+               end=c(15, 11, 12, 18, 26, 27, 28))
```

These and some common operations are illustrated in the upper panel of Figure 6.1 and summarized in Table 6.2.

Methods on ranges can be grouped as follows:

Intra-range methods act on each range independently. These include `flank`, `narrow`, `reflect`, `resize`, `restrict`, and `shift`, among others. An illustration is `shift`, which translates each range by the amount specified by the `shift` argument. Positive values shift to the right, negative to the left; `shift` can be a vector, with each element of the vector shifting the corresponding element of the *IRanges* instance. Here we shift all ranges to the right by 5, with the result illustrated in the middle panel of Figure 6.1.

```
> shift(ir, 5)

IRanges of length 7
    start end width
[1]    12  20     9
```

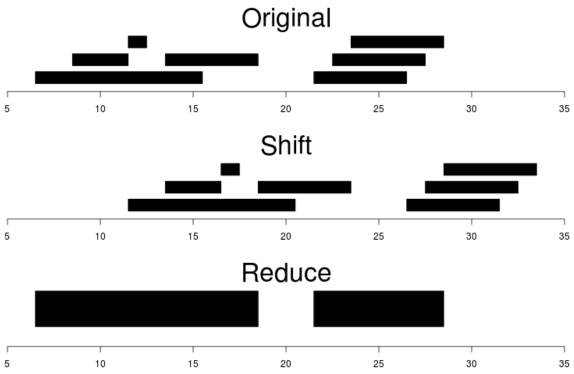


Figure 6.1: Ranges

```
[2]    14  16     3
[3]    17  17     1
[4]    19  23     5
[5]    27  31     5
[6]    28  32     5
[7]    29  33     5
```

Inter-range methods act on the collection of ranges as a whole. These include `disjoin`, `reduce`, `gaps`, and `range`. An illustration is `reduce`, which reduces overlapping ranges into a single range, as illustrated in the lower panel of Figure 6.1.

```
> reduce(ir)
```

```
IRanges of length 2
    start end width
[1]     7  18    12
[2]    22  28     7
```

`coverage` is an inter-range operation that calculates how many ranges overlap individual positions. Rather than returning ranges, `coverage` returns a compressed representation (run-length encoding)

```
> coverage(ir)
```

```
integer-Rle of length 28 with 12 runs
  Lengths: 6 2 4 1 2 3 3 1 1 3 1 1
  Values : 0 1 2 1 2 1 0 1 2 3 2 1
```

The run-length encoding can be interpreted as 'a run of length 6 of nucleotides covered by 0 ranges, followed by a run of length 2 of nucleotides covered by 1 range. . . '.

Between methods act on two (or sometimes more) *IRanges* instances. These include `intersect`, `setdiff`, `union`, `pintersect`, `psetdiff`, and `punion`.

The `countOverlaps` and `findOverlaps` functions operate on two sets of ranges. `countOverlaps` takes its first argument (the `query`) and determines how many of the ranges in the second argument (the `subject`) each overlaps. The result is an integer vector with one element for each member of `query`. `findOverlaps` performs a similar operation but returns a more general matrix-like structure that identifies each pair of query / subject overlaps. Both arguments allow some flexibility in the definition of 'overlap'.

Common operations on ranges are summarized in Table 6.2.

Table 6.2: Common operations on *IRanges*, *GRanges* and *GRangesList*.

Category	Function	Description
Accessors	`start`, `end`, `width`	Get or set the starts, ends and widths
	`names`	Get or set the names
	`mcols`, `metadata`	Get or set metadata on elements or object
	`length`	Number of ranges in the vector
	`range`	Range formed from min start and max end
Ordering	`<`, `<=`, `>`, `>=`, `==`, `!=`	Compare ranges, ordering by start then width
	`sort`, `order`, `rank`	Sort by the ordering
	`duplicated`	Find ranges with multiple instances
	`unique`	Find unique instances, removing duplicates
Arithmetic	`r + x`, `r - x`, `r * x`	Shrink or expand ranges `r` by number `x`
	`shift`	Move the ranges by specified amount
	`resize`	Change width, anchoring on start, end or mid
	`distance`	Separation between ranges (closest endpoints)
	`restrict`	Clamp ranges to within some start and end
	`flank`	Generate adjacent regions on start or end
Set operations	`reduce`	Merge overlapping and adjacent ranges
	`intersect`, `union`, `setdiff`	Set operations on reduced ranges
	`pintersect`, `punion`, `psetdiff`	Parallel set operations, on each `x[i]`, `y[i]`
	`gaps`, `pgap`	Find regions not covered by reduced ranges
	`disjoin`	Ranges formed from union of endpoints
Overlaps	`findOverlaps`	Find all overlaps for each `x` in `y`
	`countOverlaps`	Count overlaps of each `x` range in `y`
	`nearest`	Find nearest neighbors (closest endpoints)
	`precede`, `follow`	Find nearest `y` that `x` precedes or follows
	`x %in% y`	Find ranges in `x` that overlap range in `y`
Coverage	`coverage`	Count ranges covering each position
Extraction	`r[i]`	Get or set by logical or numeric index
	`r[[i]]`	Get integer sequence from `start[i]` to `end[i]`
	`subsetByOverlaps`	Subset `x` for those that overlap in `y`
	`head`, `tail`, `rev`, `rep`	Conventional R semantics
Split, combine	`split`	Split ranges by a factor into a *RangesList*
	`c`	Concatenate two or more range objects

`mcols` and `metadata` The *GRanges* class (actually, most of the data structures defined or extending those in the *IRanges* package) has two additional very useful data components. The `mcols` function allows information on each range to be stored and manipulated (e.g., subset) along with the *GRanges* instance. The element metadata is represented as a *DataFrame*, defined in *IRanges* and acting like a standard *R data.frame* but with the ability to hold more complicated data structures as columns (and with element metadata of its own, providing an enhanced alternative to the *Biobase* class *AnnotatedDataFrame*).

```
> mcols(genes) <- DataFrame(EntrezId=c("42865", "2768869"),
+                           Symbol=c("kal-1", "CG34330"))
```

`metadata` allows addition of information to the entire object. The information is in the form of a list; any data can be provided.

```
> metadata(genes) <-
+     list(CreatedBy="A. User", Date=date())
```

GRangesList The `GRanges` class is extremely useful for representing simple ranges. Some next-generation sequence data and genomic features are more hierarchically structured. A gene may be represented by several exons within it. An aligned read may be represented by discontinuous ranges of alignment to a reference. The *GRangesList* class represents this type of information. It is a list-like data structure, which each element of the list itself a *GRanges* instance. The gene FBgn0039155 contains several exons, and can be represented as a list of length 1, where the element of the list contains a *GRanges* object with 7 elements:

```
GRangesList of length 1:
$FBgn0039155
GRanges with 7 ranges and 2 metadata columns:
      seqnames               ranges strand |   exon_id   exon_name
         <Rle>            <IRanges>  <Rle> | <integer> <character>
  [1]    chr3R [19967117, 19967382]      + |     45515        <NA>
  [2]    chr3R [19970915, 19971592]      + |     45516        <NA>
  [3]    chr3R [19971652, 19971770]      + |     45517        <NA>
  [4]    chr3R [19971831, 19972024]      + |     45518        <NA>
  [5]    chr3R [19972088, 19972461]      + |     45519        <NA>
  [6]    chr3R [19972523, 19972589]      + |     45520        <NA>
  [7]    chr3R [19972918, 19973212]      + |     45521        <NA>

---
seqlengths:
    chr3R
 27905053
```

The *GRangesList* object has methods one would expect for lists (e.g., `length`, sub-setting). Many of the methods introduced for working with *IRanges* are also available, with the method applied element-wise.

The *GenomicFeatures* package Many public resources provide annotations about genomic features. For instance, the UCSC genome browser maintains the 'knownGene' track of established exons, transcripts, and coding sequences of many model organisms. The *GenomicFeatures* package provides a way to retrieve, save, and query these resources. The underlying representation is as sqlite data bases, but the data are available in *R* as *GRangesList* objects. The following exercise explores the *GenomicFeatures* package and some of the functionality for the *IRanges* family introduced above.

Exercise 9
Load the TxDb.Dmelanogaster.UCSC.dm3.ensGene annotation package, and create an alias `txdb` *pointing to the TranscriptDb object this class defines.*

Extract all exon coordinates, organized by gene, using `exonsBy`*. What is the class of this object? How many elements are in the object? What does each element correspond to? And the elements of each element? Use* `elementLengths` *and* `table` *to summarize the number of exons in each gene, for instance, how many single-exon genes are there?*

Select just those elements corresponding to flybase gene ids FBgn0002183, FBgn0003360, FBgn0025111, and FBgn0036449. Use `reduce` *to simplify gene models, so that exons that overlap are considered 'the same'.*

Solution:

```
> library(TxDb.Dmelanogaster.UCSC.dm3.ensGene)
> txdb <- TxDb.Dmelanogaster.UCSC.dm3.ensGene # alias
> ex0 <- exonsBy(txdb, "gene")
> head(table(elementLengths(ex0)))

   1    2    3    4    5    6 
3182 2608 2070 1628 1133  886 

> ids <- c("FBgn0002183", "FBgn0003360", "FBgn0025111", "FBgn0036449")
> ex <- reduce(ex0[ids])
```

6.2 Alignments and the *Rsamtools* Package

Most down-stream analysis of short read sequences is based on reads aligned to reference genomes.

Alignment formats Most main-stream aligners produce output in SAM (text-based) or BAM format. A SAM file is a text file, with one line per aligned read, and fields separated by tabs. Here is an example of a single SAM line, split into fields.

```
> fl <- system.file("extdata", "ex1.sam", package="Rsamtools")
> strsplit(readLines(fl, 1), "\t")[[1]]

 [1] "B7_591:4:96:693:509"
 [2] "73"
 [3] "seq1"
 [4] "1"
 [5] "99"
 [6] "36M"
 [7] "*"
 [8] "0"
 [9] "0"
[10] "CACTAGTGGCTCATTGTAAATGTGTGGTTTAACTCG"
[11] "<<<<<<<<<<<<<<<;<<<<<<<<<5<<<<<;:<;7"
[12] "MF:i:18"
[13] "Aq:i:73"
[14] "NM:i:0"
[15] "UQ:i:0"
[16] "H0:i:1"
[17] "H1:i:0"
```

Fields in a SAM file are summarized in Table 6.3. We recognize from the FASTQ file the identifier string, read sequence and quality. The alignment is to a chromosome 'seq1' starting at position 1. The strand of

Table 6.3: Fields in a SAM record. From http://samtools.sourceforge.net/samtools.shtml

Field	Name	Value
1	QNAME	Query (read) NAME
2	FLAG	Bitwise FLAG, e.g., strand of alignment
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost POSition of sequence
5	MAPQ	MAPping Quality (Phred-scaled)
6	CIGAR	Extended CIGAR string
7	MRNM	Mate Reference sequence NaMe
8	MPOS	1-based Mate POSition
9	ISIZE	Inferred insert SIZE
10	SEQ	Query SEQuence on the reference strand
11	QUAL	Query QUALity
12+	OPT	OPTional fields, format TAG:VTYPE:VALUE

alignment is encoded in the 'flag' field. The alignment record also includes a measure of mapping quality, and a CIGAR string describing the nature of the alignment. In this case, the CIGAR is 36M, indicating that the alignment consisted of 36 `M`atches or mismatches, with no indels or gaps; indels are represented by `I` and `D`; gaps (e.g., from alignments spanning introns) by `N`.

BAM files encode the same information as SAM files, but in a format that is more efficiently parsed by software; BAM files are the primary way in which aligned reads are imported in to *R*.

Aligned reads in *R* The `readGappedAlignments` function from the *GenomicRanges* package reads essential information from a BAM file in to *R*. The result is an instance of the *GappedAlignments* class. The *GappedAlignments* class has been designed to allow useful manipulation of many reads (e.g., 20 million) under moderate memory requirements (e.g., 4 GB).

```
> alnFile <- system.file("extdata", "ex1.bam", package="Rsamtools")
> aln <- readGappedAlignments(alnFile)
> head(aln, 3)
```

```
GappedAlignments with 3 alignments and 0 metadata columns:
      seqnames strand       cigar    qwidth     start       end     width
         <Rle>  <Rle> <character> <integer> <integer> <integer> <integer>
  [1]     seq1      +         36M        36         1        36        36
  [2]     seq1      +         35M        35         3        37        35
  [3]     seq1      +         35M        35         5        39        35
           ngap
      <integer>
  [1]         0
  [2]         0
  [3]         0
  ---
  seqlengths:
   seq1 seq2
   1575 1584
```

The `readGappedAlignments` function takes an additional argument, `param`, allowing the user to specify regions of the BAM file (e.g., known gene coordinates) from which to extract alignments.

A *GappedAlignments* instance is like a data frame, but with accessors as suggested by the column names. It is easy to query, e.g., the distribution of reads aligning to each strand, the width of reads, or the cigar strings

```
> table(strand(aln))

   +    -    *
1647 1624    0

> table(width(aln))

  30   31   32   33   34   35   36   38   40
   2   21    1    8   37 2804  285    1  112

> head(sort(table(cigar(aln)), decreasing=TRUE))

     35M      36M      40M      34M      33M 14M4I17M
    2804      283      112       37        6        4
```

Exercise 10
Use `bigdata`*,* `file.path` *and* `dir` *to obtain file paths to the BAM files. These are a subset of the aligned reads, overlapping just four genes.*

Input the aligned reads from one file using `readGappedAlignments`*. Explore the reads, e.g., using* `table` *or* `xtabs`*, to summarize which chromosome and strand the subset of reads is from.*

The object `ex` *created earlier contains coordinates of four genes. Use* `countOverlaps` *to first determine the number of genes an individual read aligns to, and then the number of uniquely aligning reads overlapping each gene. Since the RNAseq protocol was not strand-sensitive, set the strand of* `aln` *to* `*`*.*

Write the sequence of steps required to calculate counts as a simple function, and calculate counts on each file. On Mac or Linux, can you easily parallelize this operation?

Solution: We discover the location of files using standard *R* commands:

```
> bigdata <- system.file("bigdata", package="SequenceAnalysisData")
> fls <- dir(file.path(bigdata, "bam"), ".bam$", full=TRUE) #$
> names(fls) <- sub("_.*", "", basename(fls))
```

Use `readGappedAlignments` to input data from one of the files, and standard *R* commands to explore the data.

```
> ## input
> aln <- readGappedAlignments(fls[1])
> xtabs(~seqnames + strand, as.data.frame(aln))

        strand
seqnames    +    -
   chr3L 5402 5974
   chrX  2278 2283
```

To count overlaps in regions defined in a previous exercise, load the regions.

```
> data(ex)              # from an earlier exercise
```

Many RNA-seq protocols are not strand aware, i.e., reads align to the plus or minus strand regardless of the strand on which the corresponding gene is encoded. Adjust the strand of the aligned reads to indicate that the strand is not known.

```
> strand(aln) <- "*"   # protocol not strand-aware
```

One important issue when counting reads is to make sure that the reference names in both the annotation and the read files are identical.

Exercise 11
Check the reference name in both the `ex` *and* `aln`*. If they are not similar, how could you correct them?*

For simplicity, we are interested in reads that align to only a single gene. Count the number of genes a read aligns to...

```
> hits <- countOverlaps(aln, ex)
> table(hits)
```

```
hits
    0     1     2 
  772 15026   139 
```

and reverse the operation to count the number of times each region of interest aligns to a uniquely overlapping alignment.

```
> cnt <- countOverlaps(ex, aln[hits==1])
```

A simple function for counting reads (see Chapter 9 and `GenomicRangese::summarizeOverlaps` for greater detail) is

```
> counter <-
+     function(filePath, range)
+ {
+     aln <- readGappedAlignments(filePath)
+     strand(aln) <- "*"
+     hits <- countOverlaps(aln, range)
+     countOverlaps(range, aln[hits==1])
+ }
```

This can be applied to all files using `sapply`

```
> counts <- sapply(fls, counter, ex)
```

The counts in one BAM file are independent of counts in another BAM file. This encourages us to count reads in each BAM file in parallel, decreasing the length of time required to execute our program. On Linux and Mac OS, a straight-forward way to parallelize this operation is:

```
> if (require(parallel))
+     simplify2array(mclapply(fls, counter, ex))
```

Chapter 7

Strategies for working with large data

Bioinformatics data is now very large; it is not reasonable to expect all of a fastq or bam file, for instance, to fit into memory. How is this data to be processed? This challenge confronts us in whatever language or tool we are using. In *R* and *Bioconductor*, three main approaches are to: (1) *restrict* data input to the interesting subset of the larger data set; (2) *sample* from the large data, knowing that an appropriately sized sample will accurately estimate statistics we are interested in; (3) *iterate* through large data in chunks; and (4) use *parallel* evaluation on one or several computers. Let's look at each of these approaches.

7.1 Restriction

Just because a data file contains a lot of data does not mean that we are interested in all of it. In base *R*, one might use the `colClasses` argument to `read.delim` or similar function (e.g., setting some elements to `NULL`) to read only some columns of a large comma-separated value file. In addition to the obvious benefit of using less memory than if all of the file had been read in, input will be substantially faster because less computation needs to be done to coerce values from their representation in the file to their representation in *R*'s memory.

A variation on the idea of restricting data input is to organize the data on disk into a representation that facilitates restriction. In base *R*, large data might be stored in a relational data base like the *sqlite* data bases that are built in to *R* and used in the *AnnotationDbi* *Bioconductor* packages. In addition to facilitating restriction, these approaches are typically faster than parsing a plain text file, because the data base software has stored data in a way that efficiently transforms from on-disk to in-memory representation.

Let's use BAM files and the *Rsamtools* package to illustrate restriction. Rather than using simple text files ("sam" format), we use "bam" (binary alignment) files that have been indexed. Use of a binary format enhances data input speed, while the index facilitates restrictions that take into account genomic coordinates. The basic approach will use the `ScanBamParam` function to specify a restriction.

A BAM record can contain a lot of information, including the relatively large sequence, quality, and query name strings. Not all information in the BAM file is needed for some calculations. For instance, one could calculate coverage (number of nucleotides overlapping each reference position) using only the `rname` (reference sequence name), `pos`, and `cigar` fields. We could arrange to input this just information with

```
> param <- ScanBamParam(what=c("rname", "pos", "cigar"))
```

Another common restriction is to particular genomic regions, for instance known genes in an RNAseq differential expression study or to promoter regions in a ChIP-seq study. Restrictions in genome space are specified using `GRanges` objects (typically computed from some reference source, rather than entered by hand) provided as the `which` argument to `ScanBamParam`. Here we create a `GRanges` instance representing all exons on *D. melanogaster* chromosome 3L, and use that as a restriction to `ScanBamParam`'s `which` argument:

```
> library(TxDb.Dmelanogaster.UCSC.dm3.ensGene)
> exByGn <- exonsBy(TxDb.Dmelanogaster.UCSC.dm3.ensGene, "gene")
> seqlevels(exByGn, force=TRUE) <- "chr3L"
> gns <- unlist(range(exByGn))
> param <- ScanBamParam(which=gns)
```

The `what` and `which` restrictions can be combined with other restrictions into a single `ScanBamParam` object

```
> param <- ScanBamParam(what=c("rname", "pos", "cigar"), which=gns)
```

We can also restrict input to, e.g., paired-end reads that represent the primary (best) alignment; see the help page `?ScanBamParam` for a more complete description.

Reaching ahead a little bit, here are some BAM files from an experiment in *D. melanogaster*; the BAM files contain a subset of aligned reads

```
> bigdata <- system.file("bigdata", package="SequenceAnalysisData")
> bamfls <- dir(file.path(bigdata, "bam"), ".bam$", full=TRUE) #$
> names(bamfls) <- sub("_.*", "", basename(bamfls))
```

We'll read in the first BAM file, but restrict input to "rname" to find how many reads map to each chromosome. We use the "low-level" function `scanBam` to input the data

```
> param <- ScanBamParam(what="rname")
> bam <- scanBam(bamfls[1], param=param)[[1]]
> table(bam$rname)

  chr2L   chr2R   chr3L   chr3R    chr4    chrM    chrX chrYHet 
      0       0   11376       0       0       0    4561       0 
```

Restriction is such a useful concept that many *Bioconductor* high throughput sequence analysis functions enable doing the right thing. Functions such as `coverage,BamFile-method` use restrictions to read in the specific data required for them to compute the statistic of interest. Most "higher level" functions have a `param=ScanBamParam()` argument. For instance, the primary user-friendly function for reading BAM files is `readGappedAlignments` (this is renammed to `readGAlignments` in *Bioconductor* version 2.13) reads the most useful information, allowing the user to specify additional fields if desired.

```
> gal <- readGappedAlignments(bamfls[1])
> head(gal, 3)

GappedAlignments with 3 alignments and 0 metadata columns:
      seqnames strand       cigar    qwidth     start       end     width
         <Rle>  <Rle> <character> <integer> <integer> <integer> <integer>
  [1]    chr3L      - 20M498300N25M        45   1613818   2112162    498345
  [2]    chr3L      -  8M498300N37M        45   1613830   2112174    498345
  [3]    chr3L      -  8M498300N37M        45   1613830   2112174    498345
           ngap
      <integer>
  [1]         1
  [2]         1
  [3]         1
  ---
  seqlengths:
      chr2L    chr2R    chr3L    chr3R     chr4     chrM     chrX  chrYHet
   23011544 21146708 24543557 27905053  1351857    19517 22422827   347038
```

```
> param <- ScanBamParam(what="seq")  ## also input sequence
> gal <- readGappedAlignments(bamfls[1], param=param)
> head(mcols(gal)$seq)
```

```
  A DNAStringSet instance of length 6
    width seq
[1]    45 ATGTGTAGCGGGGGCACTAGCACCAGAAGCAAGGACAAACCCGCA
[2]    45 CGAACTAGCACCAGAATCAAGGACAAACCCGCAGTTGTCCATCAC
[3]    45 AGCTCTAGCACCAGAATCAAGGACAAACCCGCAGTTGTCCATCAC
[4]    45 GCGCGCAGGTGTGTGAGGAAGACCAGGGTGTTGTTGTCCTCGGGA
[5]    45 GCGCGCAGGTGTGTGAGGAAGACCAGGGTGTTGTTGTCCTCGTGT
[6]    45 CGCAGGTGTGTGAGGAAGACCAGGGTGTTGTTGTCCTCGAGATCG
```

7.2 Sampling

R is after all a statistical language, and it sometimes makes sense to draw inferences from a sample of large data. For instance, many quality assessment statistics summarize overall properties (e.g., GC content or per-nucleotide base quality of FASTQ reads) that don't require processing of the entire data. For these statistics to be valid, the sample from the file needs to be a random sample, rather than a sample of convenience.

There are two advantages to sampling from a FASTQ (or BAM) file. The sample uses less memory than the full data. And because less data needs to be parsed from the on-disk to in-memory representation the input is faster.

The *ShortRead* package `FastqSampler` (see also `Rsamtools::BamSampler` visits a FASTQ file but retains only a random sample from the file. Here is our function to calculate GC content from a `DNAStringSet`:

```
> gcFunction <-
+     function(x)
+ {
+     alf <- alphabetFrequency(x, as.prob=TRUE)
+     rowSums(alf[,c("G", "C")])
+ }
```

and a subset of a FASTQ file with 1 million reads:

```
> bigdata <- system.file("bigdata", package="SequenceAnalysisData")
> fqfl <- dir(file.path(bigdata, "fastq"), ".fastq$", full=TRUE) #$
```

`FastqSampler` works by specifying the file name and desired sample size, e.g., 100,000 reads. This creates an object from which an independent sample can be drawn using the `yield` function.

```
> sampler <- FastqSampler(fqfl, 100000)
> fq <- yield(sampler)                      # 100,000 reads
> lattice::densityplot(gcFunction(sread(fq)), plot.points=FALSE)
> fq <- yield(sampler)                      # a different 100,000 reads
```

Generally, one would choose a sample size large enough to adequately characterize the data but not so large as to consume all (or a fraction, see section 7.4 below) of the memory. The default (1 million) is a reasonable starting point.

`FastqSampler` relies on the *R* random number generator, so the same sequence of reads can be sampled by using the same random number seed. This is a convenient way to sample the same read pairs from the FASTQ files typically used to represent paired-end reads

```
> ## NOT RUN
> set.seed(123)
> end1 <- yield(FastqSampler("end_1.fastq"))
> set.seed(123)
> end2 <- yield(FastqSampler("end_2.fastq"))
```

7.3 Iteration

Restriction may not be enough to wrestle large data down to size, and sampling may be inappropriate for the task at hand. A solution is then to iterate through the file. An example in base *R* is to open a file connection, and then read and process successive chunks of the file, e.g., reading chunks of 10000 lines

```
> ## NOT RUN
> con <- file("<hypothetical-file>.txt")
> open(f)
> while (length(x <- readLines(f, n=10000))) {
+     ## work on character vector 'x'
+ }
> close(f)
```

The pattern `length(x <- readLines(f, n=10000))` is a convenient short-hand pattern that reads the *next* 10000 lines into a variable `x` and then asks `x` it's length. `x` is created in the calling environment (so it is available for processing in the `while` loop) When there are no lines left to read, `length(x)` will evaluate to zero and the `while` loop will end.

Iteration really involves three steps: input of a 'chunk' of the data; calculation of a desired summary; and aggregation of summaries across chunks. We illustrate this with BAM files in the *Rsamtools* package; see also `FastqStreamer` in the *ShortRead* package and `TabixFile` for iterating through large VCF files (via `readVCF` in the *VariantAnnotation* package).

The first stage in iteration is to arrange for input of a chunk of data. In many programming languages one would iterate 'record-at-a-time', reading in one record, processing it, and moving to the next. *R* is not efficient when used in this fashion. Instead, we want to read in a larger chunk of data, typically as much as can comfortably fit in the available memory. In *Rsamtools* for reading BAM files we arrange for this by creating a `BamFile` (or `TabixFile` for VCF) object where we specify an appropriate `yieldSize`. Here we go for a bigger BAM file

```
> library(RNAseqData.HeLa.bam.chr14)
> bamfl <- RNAseqData.HeLa.bam.chr14_BAMFILES[1]
> countBam(bamfl)

  space start end width                file records nucleotides
1    NA    NA  NA    NA ERR127306_chr14.bam  800484    57634848

> bf <- BamFile(bamfl, yieldSize=200000) # could be larger, e.g., 2 million
```

A `BamFile` object can be used to read data from a BAM file, e.g., using `readGappedAlignments`. Instead of reading all the data, the reading function will read just `yieldSize` records. The idea then is to open the BAM file and iterate through until no records are input. The pattern is

```
> ## initialize, e.g., for step 3 ...
> open(bf)
> while (length(gal <- readGappedAlignments(bf))) {
+     ## step 2: do work...
+     ## step 3: aggregate results...
+ }
> close(bf)
```

The second step is to perform a useful calculation on the chunk of data. This is particularly easy to do if chunks are independent of one another. For instance, a common operation (see chapter 9) is to count the number of times reads overlap regions of interest. There are functions that implement a variety of counting modes (see, e.g., summarizeOverlaps in the *GenomicRanges* package); here we'll go for a simple counter that arranges to tally one 'hit' each time a read overlaps (in any way) at most one range. Here is our counter function, taking as arguments a GRanges or GRangesList object representing the regions for which counts are desired, and a GappedAlignments object representing reads to be counted:

```
> counter <-
+     function(query, subject, ..., ignore.strand=TRUE)
+     ## query: GRanges or GRangesList
+     ## subject: GappedAlignments
+ {
+     if (ignore.strand)
+         strand(subject) <- "*"
+     hits <- countOverlaps(subject, query)
+     countOverlaps(query, subject[hits==1])
+ }
```

To set this step up a little more completely, we need to know the regions over which counting is to occur. Here we retrieve exons grouped by gene for the genome to which the BAM files were aligned:

```
> library(TxDb.Hsapiens.UCSC.hg19.knownGene)
> query <- exonsBy(TxDb.Hsapiens.UCSC.hg19.knownGene, "gene")
```

Thus we'll add the following lines to our loop:

```
> ## initialize, e.g., for step 3 ...
> open(bf)
> while (length(gal <- readGappedAlignments(bf))) {
+     ## step 2: do work...
+     count0 <- counter(query, gal, ignore.strand=TRUE)
+     ## step 3: aggregate results...
+ }
> close(bf)
```

It is worth asking whether the 'do work' step will always be as straight-forward. The current example is easy because counting overlaps of one read does not depend on other reads, so the chunks can be processed independently of one another. *FIXME: These are paired-end reads, so counting is* not *this easy! See chapter 9.*

The final step in iteration is to aggregate results across chunks. In our present case, counter always returns an integer vector with length(query) elements. We want to add these over chunks, and we can arrange to do this by starting with an initial vector of counts counts <- integer(length(query)) and simply adding count0 at each iteration. The complete iteration, packaged as a function to facilitate re-use is

```
> counter1 <-
+     function(bf, query, ...)
+ {
+     ## initialize, e.g., for step 3 ...
+     counts <- integer(length(query))
+     open(bf)
+     while (length(gal <- readGappedAlignments(bf, ...))) {
+         ## step 2: do work...
+         count0 <- counter(query, gal, ignore.strand=TRUE)
+         ## step 3: aggregate results...
+         counts <- counts + count0
+     }
+     close(bf)
+     counts
+ }
```

In action, this is invoked as

```
> bf <- BamFile(bamfl, yieldSize=500000)
> counts <- counter1(bf, query)
```

Counting is a particularly simple operation; one will often need to think carefully about how to aggregate statistics derived from individual chunks. A useful general approach is to identify *sufficient statistics* that represent a sufficient description of the data and can be easily aggregated across chunks. This is the approach taken by, for instance, the *biglm* package for fitting linear models to large data sets – the linear model is fit to successive chunks and the fit reduced to sufficient statistics, the sufficient statistics are then added across chunks to arrive at an overall fit.

7.4 Parallel evaluation

Each of the preceding sections addressed memory management; what about overall performance?

The starting point is really writing efficient, vectorized code, with common strategies outlined in Chapter 2. Performance differences between poorly written versus well written *R* code can easily span two orders of magnitude, whereas parallel processing can only increase throughput by an amount inversely proportional to the number of processing units (e.g., CPUs) available.

The memory management techniques outlined earlier in this chapter are important in a parallel evaluation context. This is because we will typically be trying to exploit multiple processing cores on a single computer, and the cores will be competing for the same pool of shared memory. We thus want to arrange for the collection of processors to cooperate in dividing available memory between them, i.e., each processor needs to use only a fraction of total memory.

There are a number of ways in which *R* code can be made to run in parallel. The least painful and most effective will use 'multicore' functionality provided by the *parallel* package; the *parallel* package is installed by default with base *R*, and has a useful vignette [21]. Unfortunately, multicore facilities are not available on Windows computers.

Parallel evaluation on several cores of a single Linux or MacOS computer is particularly easy to achieve when the code is already vectorized. The solution on these operating systems is to use the functions `mclapply` or `pvec`. These functions allow the 'master' process to 'fork' processes for parallel evaluation on each of the cores of a single machine. The forked processes initially share memory with the master process, and only make copies when the forked process modifies a memory location ('copy on change' semantics). On Linux and MacOS, the `mclapply` function is meant to be a 'drop-in' replacement for `lapply`, but with iterations being evaluated on different cores. The following illustrates the use of `mclapply` and `pvec`, for a toy vectorized function `f`:

```
> library(parallel)
> f <- function(i) {
+     cat("'f' called, length(i) = ", length(i), "\n")
+     sqrt(i)
+ }
> res0 <- mclapply(1:5, f, mc.cores=2)
```

```
'f' called, length(i) =  1
'f' called, length(i) =  1
```

```
'f' called, length(i) =  1
'f' called, length(i) =  1
'f' called, length(i) =  1
```

```
> res1 <- pvec(1:5, f, mc.cores=2)
```

```
'f' called, length(i) =  3
'f' called, length(i) =  2
```

```
> identical(unlist(res0), res1)
```

```
[1] TRUE
```

`pvec` takes a vectorized function and distributes computation of different chunks of the vector across cores. Both functions allow the user to specify the number of cores used, and how the data are divided into chunks.

The *parallel* package does not support fork-like behavior on Windows, where users need to more explicitly create a cluster of *R* workers and arrange for each to have the same data loaded into memory; similarly, parallel evaluation across computers (e.g., in a cluster) require more elaborate efforts to coordinate workers; this is typically done using `lapply`-like functions provided by the *parallel* package but specialized for simple ('snow') or more robust ('MPI') communication protocols between workers.

Data movement and random numbers are two important additional considerations in parallel evaluation. Moving data to and from cores to the manager can be expensive, so strategies that minimize explicit movement (e.g., passing file names data base queries rather than *R* objects read from files; reducing data on the worker before transmitting results to the manager) can be important. Random numbers need to be synchronized across cores to avoid generating the same sequences on each 'independent' computation.

How might parallel evaluation be exploited in *Bioconductor* work flows? One approach when working with BAM files exploits the fact that data are often organized with one sample per BAM file. Suppose we are interested in running our iterating counter `counter1` over several BAM files. We could do this by creating a `BamFileList` with appropriate `yieldSize`

```
> fls <- RNAseqData.HeLa.bam.chr14_BAMFILES # 8 BAM files
> bamfls <- BamFileList(fls, yieldSize=500000) # yieldSize can be larger
```

and using an `lapply` to take each file in turn and performing the count.

```
> counts <- simplify2array(lapply(bamfls, counter1, query))
```

The parallel equivalent of this is simply (note the change from `lapply` to `mclapply`)

```
> options(mc.cores=detectCores())          # use all cores
> counts <- simplify2array(mclapply(bamfls, counter1, query))
> head(counts[rowSums(counts) != 0,], 3)
```

```
          ERR127306 ERR127307 ERR127308 ERR127309 ERR127302 ERR127303 ERR127304
10001           207       277       217       249       303       335       362
100128927       572       629       597       483       379       319       388
100129075        62        70        56        63        34        51        88
          ERR127305
10001           300
100128927       435
100129075        79
```

7.5 And. . .

The *foreach* package can be useful for parallel evaluation written using coding styles more like `for` loops rather than `lapply`. The *iterator* package is an abstraction that simplifies the notion of iterating over objects. The *Rmpi* package provides access to MPI, a structured environment for calculation on clusters. The *pbdR* formalism[1] is especially useful for well-structured distributed matrix computations.

The *MatrixEQTL* package is an amazing example implementing high performance algorithms on large data; the corresponding publication [26] is well worth studying.

Bioconductor provides an Amazon machine instance with MPI and *Rmpi* installed[2]. This can be an effective way to gain access to large computing resources.

[1] https://rdav.nics.tennessee.edu/2012/09/pbdr/

[2] http://bioconductor.org/help/bioconductor-cloud-ami/

Part II

Differential Representation

Chapter 8

RNA-seq Work Flows

8.1 Varieties of RNA-seq

RNA-seq experiments typically ask about differences in transcription of genes or other features across experimental groups. The analysis of designed experiments is statistical, and hence an ideal task for *R*. The overall structure of the analysis, with tens of thousands of features and tens of samples, is reminiscent of microarray analysis; some insights from the microarray domain will apply, at least conceptually, to the analysis of RNA-seq experiments.

The most straight-forward RNA-seq experiments quantify abundance for known gene models. The known models are derived from reference databases, reflecting the accumulated knowledge of the community responsible for the data. The 'knownGenes' track of the UCSC genome browser represents one source of such data. A track like this describes, for each gene, the transcripts and exons that are expected based on current data. The *GenomicFeatures* package allows ready access to this information by creating a local database out of the track information. This data base of known genes is coupled with high throughput sequence data by counting reads overlapping known genes and modeling the relationship between treatment groups and counts.

A more ambitious approach to RNA-seq attempts to identify novel transcripts. This requires that sequenced reads be assembled into contigs that, presumably, correspond to expressed transcripts that are then located in the genome. Regions identified in this way may correspond to known transcripts, to novel arrangements of known exons (e.g., through alternative splicing), or to completely novel constructs. We will not address the identification of completely novel transcripts here, but will instead focus on the analysis of the designed experiments: do the transcript abundances, novel or otherwise, differ between experimental groups?

8.2 Work flows and upstream analysis

RNA-seq work flows aim at measuring gene expression through assessment of mRNA abundance. Work flows involve:

1. Experimental design.
2. Wet-lab protocols for mRNA extraction and reverse transcription to cDNA.
3. Sequencing.
4. Alignment of sequenced reads to a reference genome.
5. Summarizing of the number of reads aligning to a region.
6. Normalization of samples to accommodate purely technical differences in preparation.
7. Statistical assessment of differential representation, including specification of an appropriate error model.
8. Interpretation of results in the context of original biological questions.

The inference is that higher levels of gene expression translate to more abundant cDNA, and greater numbers of reads aligned to the reference genome. The enumeration above seems simplistic, but oddly enough one has concerns and commentary on each point.

8.2.1 Experimental design

Obviously one should follow best practices for designing experiments appropriate for the data under analysis. A typical experiment will have one or several groups. Because there is uncertainty in each measurement, we require replication. Previous work shows that technical replication (repeating identical wet-lab and sequencing protocols on a single biological sample) introduces variation that is small [15] compared to biological replicates (using different samples). Most RNA-seq experiments require biological replication, and seldom include technical replicates.

How many biological replicates? It is helpful to think in terms of orders of magnitude – biological treatments with strong and consistent consequences for gene expression will be detected with a handful – 2 or 3 – replicates per treatment. Conversely, statistically subtle effects will not be much revealed by samples of say 5 or 8, but will instead require 10's or 100's of samples.

How complicated an experimental design? The advice must be to 'keep it simple'. There are many interesting biological questions that one could ask, but experimental designs with more than one or at most two factors, or with multiple levels per factor, will undermine statistical power and complicate analysis. There are exceptions of course, for instance a time course design or an experiment with two or more factors, but these require strong *a priori* motivation and confidence that the design is amenable to analysis even in the face of wet-lab or sequencing catastrophe.

What kind of treatment? Two 'lessons learned' from microarray analysis and applicable to RNA-seq inform this question. (a) It is necessary to normalize observations between samples to accommodate purely technical variation in overall patterns of expression. For example, samples provided to the sequencer have different amounts of DNA, resulting in variation in total numbers of sequenced and aligned reads independent of any difference in gene-level differential representation. This implies that the treatment should *affect only a fraction of the genes assayed*, otherwise treatment effects and protocol artifacts are confounded. (b) Between-gene measures of expression differ for reasons unrelated to levels of expression. For instance, standard protocols mean that a long gene is sequenced more often than a short gene, even when the number of mRNA molecules of the two genes are identical. This means that the most productive approach to differential representation will *compare genes across samples*, rather than compare levels of representation of different genes (gene set enrichment analysis and other approaches to between-gene comparison are statistically interesting in part because of the need to overcome between-gene differences arising for purely technical reasons). The combination of lessons (a) and (b) dictate that the treatment should affect only a subset of the genes under study, and that 'interesting' results correspond to treatment groups with differences at the gene level. *A priori* motivation, e.g., about well-defined pathways as targets of differential representation, may trump part (b) of this guideline.

The reality of executing designed experiments may mean that there are known but unavoidable factors that confound the analysis, but that are not of fundamental biological interest. Perhaps samples are being processed by different groups, or processing is spread over several months to accommodate personnel or sequencer availability. It is essential to avoid confounding such factors with biologically relevant parts of the experiment. Having acknowledged a potentially confounding factor, what is to be done? A first reaction might be randomization – arrange for samples to be processed in a random order, for instance, rather than by treatment group – but a better strategy is usually to include a blocking factor, e.g., processed by lab 'A' versus lab 'B' and to ensure that treatments are represented by replicates in each blocking factor. The down-stream analysis can then use replication to statistically accommodate such effects.

8.2.2 Wet-lab protocols, sequencing, and alignment

The important point here is that wet-lab protocols, sequencing reactions, and alignment introduce artifacts that need to be acknowledged and, if possible, accommodated in down-stream analysis. These artifacts and

Table 8.1: Statistical issues in RNA-seq differential expression.

Analysis stage	Issues
Summarizing	Counts versus RPKM and other summaries.
Normalization	Robust estimates of library size.
Differential expression	Appropriate error model (Negative Binomial, Poisson, ...); dispersion (under negative binomial) as parameter requiring estimation; ‘shrinkage’ to balance accuracy of per-gene estimates with precision of experiment-wide estimates.
Testing	Filtering to reduce multiple comparisons & false discovery rate.

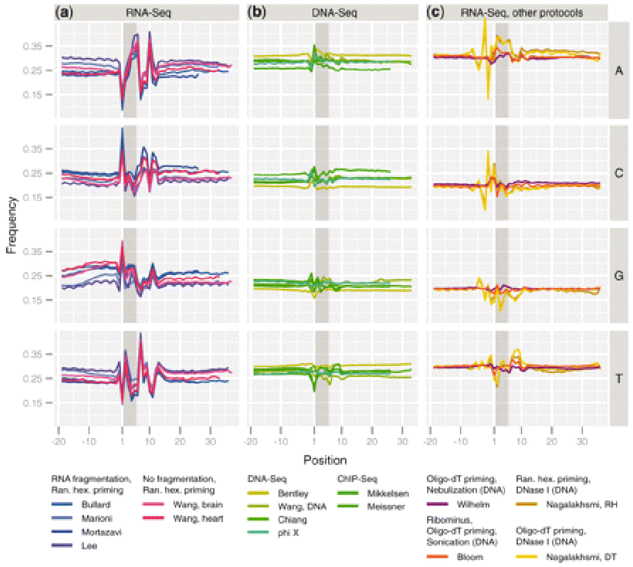


Figure 8.1: Nucleotide frequency versus position relative to start of alignment, various experiments and protocols; see [10].

approaches to their remediation are discussed in the following sections.

8.3 Statistical analysis

Important statistical issues are summarized in Table 8.1.

8.3.1 Summarizing

The Summarizing process tallies the number of reads aligning in each region (e.g., gene) of interest. The simplest method is to simply count reads overlapping each region, dividing by the length of the region of interest to accommodate differences in gene length. This is the ‘RPKM’ (reads per kilobase per million reads) of Mortazavi et al. [18]. One problem with this approach is that reads are not sampled uniformly across genes (Figure 8.1; [10]), so gene length (the ‘PK’ part of RPKM) is not a good proxy for expression level.

More fundamentally, each read represents an observation, and contributes to the certainty with which a gene is measured as ‘expressed’. A summary measure like RPKM fails to incorporate uncertainty – a particular value of RPKM might result from alignment of one or 100 reads. This contrasts with a simple count of the number of reads in the region of interest. Furthermore, count data has known statistical properties that can be exploited in down-stream statistical analysis. Thus the result of summarization most useful for assessing differential expression is read count.

How to count? For instance, should a read that partly overlaps a 5' UTR or an intron be included in a tally? What about reads that overlap multiple genes? This is a non-trivial question because alignment is only approximate (reflecting sequencing and other biases) and because sample preparation protocols and organism biology (e.g., whether the UTR or fully mature mRNA is sequenced) may dictate particular counting strategies; more elaborate counting strategies might be entertained for paired end reads. Anders enumerates some counting strategies[1]; these are implemented in his *HTSeq* python scripts, in `summarizeOverlaps` in the *GenomicRanges* package, or in functions in the (linux-only) *Rsubread* and *gmapR* packages.

8.3.2 Normalization

Normalization arises from the need to correct for purely technical differences between samples. The most common symptom of the need for normalization is differences in the total number of aligned reads. The 'M' part of RPKM measure mentioned in the context of summarization is one way of normalizing for total count. This normalization is not appropriate, because the distribution of aligned reads across genes within a sample is not uniform – some regions receive many more alignments than do others – and this distribution may differ between samples.

The overall strategy with normalization is to choose an appropriate baseline, and express sample counts relative to that baseline. There are several approaches to choice of appropriate baseline. One might choose total count for normalization, but this is a poor choice when one or a few regions of interest are very well represented – we are normalizing to the well-represented genes rather than to sequencing depth in each sample. Other straight-forward approaches include use of house-keeping genes, or the expression level from a particular quantile of the distribution of gene expression values of each sample [4]. One might attempt a robust estimate of sample abundance that is less sensitive to extreme outliers, e.g., the trimmed geometric mean of counts [1]. Another approach is TMM [22], which measures the trimmed mean of M and A values (M values are the log fold change in the number of reads aligning to a region of interest measured relative to an average or arbitrary sample, A is the average count of a gene; the trimmed mean discards regions of interest that have extreme M or A values and calculates the mean M value of the remainder); the inverse of this mean is used to weight samples. More data-driven approaches exploiting the gene-specific properties include conditional quantile normalization (implemented in the *cqn* package; [11]).

Another approach to normalization, increasingly popular as experiment size and data consistency increases, is to perform a data transformation and apply normalization methods developed for analysis of microarrays. Examples of this approach include `varianceStabilizingTransformation` from the *DESeq* package, and `voom` from the *limma* package; see the corresponding help pages of these functions for details).

8.3.3 Error model

1. Negative binomial error model often appropriate – combination of Poisson ('shot' noise, technical variation in read counts) and variation between biological samples.
2. Requires estimate of 'dispersion'
3. 'Shrinkage' of gene-specific estimates to average.
4. *DESeq*, *edgeR* take different approaches, especially to dealing with outlier / extreme values.
5. Addition modeling approaches possible, e.g., implemented in the *DSS* package [28].

8.3.4 Multiple comparison

1. Increase statistical power and reduce false discovery rate by filtering regions of interest prior to analysis.

[1] http://www-huber.embl.de/users/anders/HTSeq/doc/count.html

Table 8.2: Selected *Bioconductor* packages for RNA-seq analysis.

Package	Description
EDASeq	Exploratory analysis and QA; also *qrqc*, *ShortRead*, *DESeq*.
edgeR, *DESeq*	Generalized Linear Models using negative binomial error.
BitSeq	Bayesian inference of individual transcript abundances followed by differential expression.
DEXSeq	Exon-level differential representation.
DSS, *vsn*, *cqn*	RNA-seq normalization methodologies. Also `voom` in *limma*.
goseq	Gene set enrichment tailored to RNAseq count data; also *limma*'s `roast` or `camera` after transformation with `voom`.
easyRNASeq	Workflow; also *ArrayExpressHTS*, *rnaSeqMap*, *oneChannelGUI*.
Rsubread	Alignment (Linux only); also *gmapR*; *Biostrings* `matchPDict` for special-purpose alignments.
cummeRbund	Exploration and analysis of Cufflinks results.

2. Motivation (a): just because genes are assayed does not mean, *a priori*, that they represent something requiring a statistical test. (b) Some observations, e.g., zero counts across all samples, cannot possibly be statistically significant, independent of hypothesis under investigation.
3. Approach – detection or 'K over A'-style filter; representation of a minimum of A (normalized) read counts in at least K samples. A usually measured as counts per million. Guidelines for choice of values a little *ad hoc*; see, e.g., the *edgeR* user manual. Variance filter, e.g., IQR (inter-quartile range) provides a robust estimate of variability; can be used to rank and discard least-varying regions.

8.3.5 *Bioconductor* software

Bioconductor packages play a role in several stages of an RNA-seq analysis (Table 8.2; a more comprehensive list is under the RNAseq and HighThroughputSequencing BiocViews terms). The *GenomicRanges* infrastructure can be effectively employed to quantify known exon or transcript abundances. Quantified abundances are in essence a matrix of counts, with rows representing features and columns samples. The *edgeR* [22] and *DESeq* [1] packages facilitate analysis of this data in the context of designed experiments, and are appropriate when the questions of interest involve between-sample comparisons of relative abundance. The *DEXSeq* package extends the approach in *edgeR* and *DESeq* to ask about within-gene, between group differences in exon use, i.e., for a given gene, do groups differ in their exon use?

Chapter 9

Counting RNA-seq reads

An essential and surprisingly nuanced challenge in analyzing RNA-seq data is to actually count reads overlapping regions of interest. Issues arising during this activity appear in other aspects of high-throughput sequence analysis, so a more comprehensive navigation of the process seems to be in order. The following may appear daunting, but the short form (gene-level summary of reads overlapping exons) is as follows:

```
> ## 'Annotation' describing known gene models
> library(TxDb.Hsapiens.UCSC.hg19.knownGene)
> exByGn <- exonsBy(TxDb.Hsapiens.UCSC.hg19.knownGene, "gene")
> ## reads sorted by qname
> fls <- dir("~/some/dir", pattern="bam$")
> bamfls <- BamFileList(fls, index=character(),
+                 obeyQname=TRUE, yieldSize=1000000)
> ## restrict to primary alignment of paired reads
> flag <- scanBamFlag(isNotPrimaryRead=FALSE, isProperPair=TRUE)
> param <- ScanBamParam(flag=flag)
> ## count reads overlapping a gene model; each read counted
> ## at most once; strand-unaware assay; paired-end reads;
> cnts <- summarizeOverlaps(exByGn, bamfls, mode=Union,
+     ignore.strand=TRUE, single.end=FALSE, param=param)
```

For the long form, we use data from [29][1]. The FASTQ files are available through ArrayExpress[2]. From the abstract:

> There are 650,000 Alu elements in transcribed regions of the human genome. These elements contain cryptic splice sites, so they are in constant danger of aberrant incorporation into mature transcripts. Despite posing a major threat to transcriptome integrity, little is known about the molecular mechanisms preventing their inclusion. Here, we present a mechanism for protecting the human transcriptome from the aberrant exonization of transposable elements. Quantitative iCLIP data show that the RNA-binding protein hnRNP C competes with the splicing factor U2AF65 at many genuine and cryptic splice sites. Loss of hnRNP C leads to formation of previously suppressed Alu exons, which severely disrupt transcript function. Minigene experiments explain disease-associated mutations in Alu elements that hamper hnRNP C binding. Thus, by preventing U2AF65 binding to Alu elements, hnRNP C plays a critical role as a genome-wide sentinel protecting the transcriptome. The findings have important implications for human evolution and disease.

[1] http://europepmc.org/abstract/MED/23374342/

[2] http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1147/

9.1 ‘Phenotypic’ data

Since the data are in ArrayExpress, it is straight-forward to retrieve information about each sample:

```
> url <- sprintf("%s/%s",
+     "http://www.ebi.ac.uk/arrayexpress/files",
+     "E-MTAB-1147/E-MTAB-1147.sdrf.txt")
> pdata <- read.delim(url, sep="\t", stringsAsFactors=FALSE)
```

Some of the data available, including all-important information about factor levels in the experimental design, are summarized here:

```
> pdata[1:3, grep("Characteristics", names(pdata))]

  Characteristics.Organism. Characteristics.Genotype. Characteristics.CellLine.
1              Homo sapiens                   control                      HeLa
2              Homo sapiens                   control                      HeLa
3              Homo sapiens                   control                      HeLa
  Characteristics.Sex.
1               female
2               female
3               female
```

```
> pdata[1:3, grep("Comment", names(pdata))]

  Comment.ENA_SAMPLE. Comment.ORIENTATION. Comment.NOMINAL_LENGTH.
1           ERS140920              5-3-3-5                     175
2           ERS140920              5-3-3-5                     175
3           ERS140920              5-3-3-5                     175
  Comment.NOMINAL_SDEV. Comment.LIBRARY_LAYOUT. Comment.LIBRARY_SOURCE.
1                    25                  PAIRED          TRANSCRIPTOMIC
2                    25                  PAIRED          TRANSCRIPTOMIC
3                    25                  PAIRED          TRANSCRIPTOMIC
  Comment.LIBRARY_STRATEGY. Comment.LIBRARY_SELECTION. Comment.ENA_EXPERIMENT.
1                   RNA-Seq                     RANDOM               ERX103445
2                   RNA-Seq                     RANDOM               ERX103445
3                   RNA-Seq                     RANDOM               ERX103444
               Comment.SUBMITTED_FILE_NAME. Comment.ENA_RUN.
1 SLX-2650.CRIRUN_441.s_5.1_sequence.txt.gz        ERR127306
2 SLX-2650.CRIRUN_441.s_5.2_sequence.txt.gz        ERR127306
3 SLX-2651.CRIRUN_441.s_6.1_sequence.txt.gz        ERR127307
                                                        Comment.FASTQ_URI.
1 ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR127/ERR127306/ERR127306_1.fastq.gz
2 ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR127/ERR127306/ERR127306_2.fastq.gz
3 ftp://ftp.sra.ebi.ac.uk/vol1/fastq/ERR127/ERR127307/ERR127307_1.fastq.gz
  Comment.SPOT_LENGTH. Comment.quality_scoring_system.
1                   72                           phred
2                   72                           phred
3                   72                           phred
  Comment.quality_encoding. Comment.ascii_offset.
1                     ascii                     @
2                     ascii                     @
3                     ascii                     @
  Comment.READ_INDEX_1_BASE_CCORD.
```

```
1                                73
2                                73
3                                73
```

```
> pdata$Factor.Value.RNAI.
```

```
 [1] "control" "control" "control" "control" "control" "control" "control"
 [8] "control" "KD1"     "KD1"     "KD1"     "KD1"     "KD2"     "KD2"
[15] "KD2"     "KD2"
```

Importantly, the data are 72-mer paired-end reads.

The data include information about each FASTQ file; we simplify the data by dropping these columns and retaining unique rows. We will see that each row then corresponds to aligned reads in a BAM file.

```
> drop <- c("Scan.Name", "Comment.SUBMITTED_FILE_NAME.",
+           "Comment.FASTQ_URI.")
> pdata <- unique(pdata[,!names(pdata) %in% drop])
```

9.2 Alignments

9.2.1 Alignment, subsetting, and access in *R*

The authors provide reads as FASTQ files. These were aligned to hg19, and a subset of the aligned reads are available in the *RNAseqData.HeLa.bam.chr14* package (this package is available in *Bioconductor* version 2.13; its name may have changed). Some details of the alignment are provided in the package description:

```
> packageDescription("RNAseqData.HeLa.bam.chr14")
```

```
Package: RNAseqData.HeLa.bam.chr14
Title: Aligned reads from RNAseq experiment: Transcription profiling by
        high throughput sequencing of HNRNPC knockdown and control HeLa
        cells
Description: The package contains 8 BAM files, 1 per sequencing run.
        Each BAM file was obtained by (1) aligning the reads
        (paired-end) to the full hg19 genome with TopHat2, and then (2)
        subsetting to keep only alignments on chr14. See accession
        number E-MTAB-1147 in the ArrayExpress database for details
        about the experiment, including links to the published study
        (by Zarnack et al., 2012) and to the FASTQ files.
URL: http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1147/
Version: 0.1.1
Author: H. Pages
Maintainer: H. Pages <hpages@fhcrc.org>
Suggests: Rsamtools
biocViews: ExperimentData, RNAseqData, Homo_sapiens
License: LGPL
Packaged: 2013-05-23 18:01:21 UTC; biocbuild
Built: R 3.0.1; ; 2013-05-26 18:18:20 UTC; unix

-- File: /home/mtmorgan/R/x86_64-unknown-linux-gnu-library/3.0/RNAseqData.HeLa.bam.chr14/Meta/package.r
```

with a more extensive description available on the package help page

```
> package?RNAseqData.HeLa.bam.chr14
```

and included scripts

```
> README_file <- system.file("scripts", "README.TXT",
+                            package="RNAseqData.HeLa.bam.chr14")
> cat(readLines(README_file), sep="\n")
```

The end result is 8 BAM files representing reads aligned to chromosome 14, along with the BAM index files.

```
> library(RNAseqData.HeLa.bam.chr14)
> library(Rsamtools)
> fls <- RNAseqData.HeLa.bam.chr14_BAMFILES
> bfl <- BamFileList(fls)
> bfl
```

```
BamFileList of length 8
names(8): ERR127306 ERR127307 ERR127308 ... ERR127303 ERR127304 ERR127305
```

```
> bf <- bfl[[1]]; bf
```

```
class: BamFile
path: /home/mtmorgan/R/x86_64-unknown-linux-gnu-library/3.../ERR127306_chr14.bam
index: /home/mtmorgan/R/x86_64-unknown-linux-gnu-library/.../ERR127306_chr14.bam
isOpen: FALSE
yieldSize: NA
obeyQname: FALSE
```

9.2.2 Exploration

It is useful to get a sense of the data in these files. We can query one file for the information about the sequence dictionary created when the reads were aligned

```
> head(seqlengths(bfl[[1]]))
```

```
                 chr1                 chr10                 chr11
            249250621             135534747             135006516
chr11_gl000202_random                 chr12                 chr13
                40103             133851895             115169878
```

Aligners rarely record the genome to which reads were aligned, but this can often be inferred by the sequence lengths. BAM file headers commonly contain additional information, e.g., about the program and command line used in the alignment; this can be retrieved by querying the BAM file for it's header information

```
> hdr <- scanBamHeader(bfl[[1]])
```

and then exploration, e.g., to

```
> hdr[["text"]][["@PG"]][[1]]
```

```
[1] "ID:TopHat"
```

```
> substr(hdr[["text"]][["@PG"]][[3]], 1, 60)
```

```
[1] "CL:/home/hpages/tophat-2.0.8b.Linux_x86_64/tophat --mate-inn"
```

Tags such as ‘@PG’, ‘ID’, ‘CL’, etc., are described in the BAM file specification[3].

The countBam utility provides a summary of the reads and nucleotides in each BAM file; it can be invoked with a param=ScanBamParam() object to restrict the type (e.g., only proper pairs) and location (e.g., coding regions) over which counting occurs.

```
> countBam(bfl)

          space start end width                file records nucleotides
ERR127306    NA    NA  NA    NA ERR127306_chr14.bam  800484    57634848
ERR127307    NA    NA  NA    NA ERR127307_chr14.bam  875302    63021744
ERR127308    NA    NA  NA    NA ERR127308_chr14.bam  875608    63043776
ERR127309    NA    NA  NA    NA ERR127309_chr14.bam  778464    56049408
ERR127302    NA    NA  NA    NA ERR127302_chr14.bam  742754    53478288
ERR127303    NA    NA  NA    NA ERR127303_chr14.bam  786196    56606112
ERR127304    NA    NA  NA    NA ERR127304_chr14.bam  810724    58372128
ERR127305    NA    NA  NA    NA ERR127305_chr14.bam  798376    57483072
```

The quickCountBam function provides a higher-resolution summary

```
> quickCountBam(bf)

                               group |    nb of |    nb of | mean / max
                                  of |  records |   unique | records per
                             records | in group |   QNAMEs | unique QNAME
All records........................ A |   800484 |   393300 | 2.04 / 10
  o template has single segment.... S |        0 |        0 |   NA / NA
  o template has multiple segments. M |   800484 |   393300 | 2.04 / 10
      - first segment.............. F |   400242 |   393300 | 1.02 / 5
      - last segment............... L |   400242 |   393300 | 1.02 / 5
      - other segment.............. O |        0 |        0 |   NA / NA

Note that (S, M) is a partitioning of A, and (F, L, O) is a partitioning of M.
Indentation reflects this.

Details for group M:
  o record is mapped.............. M1 |   800484 |   393300 | 2.04 / 10
      - primary alignment......... M2 |   779228 |   389614 |    2 / 2
      - secondary alignment....... M3 |    21256 |    10052 | 2.11 / 8
  o record is unmapped............ M4 |        0 |        0 |   NA / NA

Details for group F:
  o record is mapped.............. F1 |   400242 |   393300 | 1.02 / 5
      - primary alignment......... F2 |   389614 |   389614 |    1 / 1
      - secondary alignment....... F3 |    10628 |    10052 | 1.06 / 4
  o record is unmapped............ F4 |        0 |        0 |   NA / NA

Details for group L:
  o record is mapped.............. L1 |   400242 |   393300 | 1.02 / 5
      - primary alignment......... L2 |   389614 |   389614 |    1 / 1
      - secondary alignment....... L3 |    10628 |    10052 | 1.06 / 4
  o record is unmapped............ L4 |        0 |        0 |   NA / NA
```

[3] http://samtools.sourceforge.net/SAM1.pdf

We can explore the reads by sampling a subset from the entire file; read sequences and mapping quality might be particularly interesting for quality assessment purposes.

```
> sampler <- BamSampler(fls[1], yieldSize=100000)
> param <- ScanBamParam(what=c("seq", "mapq"))
> gal <- readGappedAlignments(sampler, param=param)
> table(qwidth(gal))     # width of reads: all 72mers

    72
100000

> range(width(gal))      # width of _alignments_

[1]     70 196447

> table(mcols(gal)$mapq)

    0     1     3    50
   26   864  4230 94880
```

Reads in BAM files are stored from 5' to 3' on the plus strand; this means that reads mapping to the minus strand need to be reversed to retrieve their 5' orientation as produced by the sequencer. Protocol artifacts are apparent (Figure 9.1).

```
> seq <- mcols(gal)$seq
> idx <- as.logical(strand(gal) == "-")
> seq[idx] <- reverseComplement(seq[idx])
> abc <- ShortRead::alphabetByCycle(seq)[1:4,]
> matplot(t(abc), type="l", lty=1, lwd=2)
> legend("topright", legend=rownames(abc)[1:4], col=1:4, lty=1, lwd=2)
```

9.3 Counting

Counting is surprisingly nuanced. The basic principle is that each template generates one piece of evidence. A template consists of all segments derived from a single spot on the sequencing machine. Thus (1) both ends of a paired end read belong to the same template, and the pair together should only be counted once. In an RNA-seq experiment, a template can only come from a single RNA molecule. Hence (2) a template cannot be double counted if, for instance, it overlaps exons from two distinct genes or transcripts. Since the template is derived from mRNA, in measuring expression of known gene models, (3) the template should in principle be entirely consistent (e.g., aligned to share exon boundaries) with the gene model; one can certainly envision biological, protocol, and alignment reasons that might lead to imperfect agreement. Many RNA-seq protocols amplify cDNA in a way that loses track of the strand from which the original mRNA derived, thus (4) strand of alignment may or may not be important, depending on sequencing protocol. Depending on the research question of interest, (5) counting might occur over genes, or over transcripts.

How are these considerations to be taken into account? We will aim for the `summarizeOverlaps` function, and in particular the methods specialized on *BamFile* instances defined in the *Rsamtools* package. The essential arguments are `features` and `reads`

Features Features are typically *GRanges* or *GRangesList* instances. We start by loading the appropriate a 'transcriptDb' package containing information about known gene models; other sources of annotation are also available, e.g., GFF / GTF files provided by ensembl or UCSC.

```
> library(TxDb.Hsapiens.UCSC.hg19.knownGene)
```

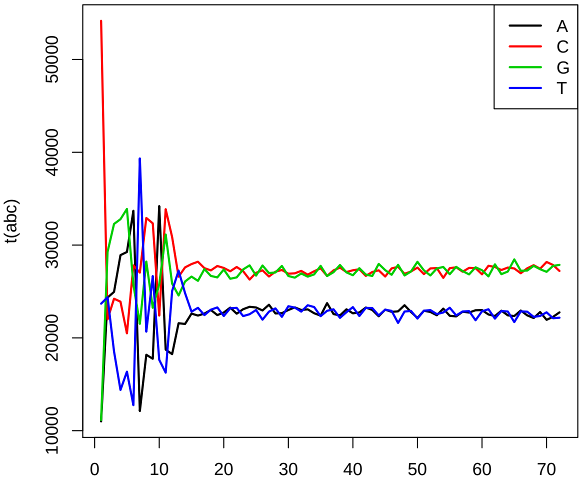


Figure 9.1: Called nucleotides as a function of cycle. Early bases are biased.

For *gene-level* differential expression, a starting point is to count tempates that overlap exons in the known gene model. For this counting model we extract from the transcriptDb all exons, organized by gene; for the analysis in this chapter we restrict the model to genes on chromosome 14.

```
> exByGn <- exonsBy(TxDb.Hsapiens.UCSC.hg19.knownGene, "gene")
> seqlevels(exByGn, force=TRUE) <- "chr14"
```

There are 764 genes on this chromosome.

Reads The first issue is that both ends of a paired end read need to be treated as one unit for counting. We can read an entire BAM file into memory and then pair reads, but this is extremely memory intensive. An alternative is to sort the BAM files by query name. Because the reads are sorted by query name, it is then easy to pair reads 'on the fly' and hence to iterate through a large file. Here we sort our reads using `sortBam` from the *Rsamtools* package.

```
> destdir <- "~/SequenceData/RNAseqData.HeLa.bam.chr14"
> if (!file.exists(destdir)) {
+     dir.create(destdir)
+     dest0 <- sub(".bam$", "_sorted", basename(fls))
+     dest <- mcmapply(sortBam, fls, file.path(destdir, dest0),
+                      MoreArgs=list(byQname=TRUE))
+ } else {
+     dest <- dir(destdir, "bam$", full=TRUE)
+     names(dest) <- sub("_.*", "", basename(dest))
+ }
> sorted <- BamFileList(dest, index=character(), obeyQname=TRUE,
+                            yieldSize=500000)
```

FIXME: Bioconductor release 2.13 will not require BAM files to be sorted in this way.

Additional parameters With features and reads in hand, we turn to additional parameters of `summarizeOverlaps`:

mode One of three well-defined counting modes, described on `?summarizeOverlaps` and pictured in the HT-Seq manual[4].

ignore.strand `TRUE` when the protocol does not preserve strand-of-origin information..

single.end `FALSE` when reads are paired-end.

param A *ScanBamParam* instance further restricting which reads are counted, e.g., proper pairs that are flagged by the aligner as the primary alignment.

```
> flag <- scanBamFlag(isNotPrimaryRead=FALSE, isProperPair=TRUE)
> param <- ScanBamParam(flag=flag)
```

Bioconductor version 2.13 introduces an additional argument

inter.feature which when `TRUE` (the default) counting is inter-feature aware and reads overlap more than one feature are NOT counted.

And finally. . . Here we summarize counts across all sorted BAM files. The counting is done in parallel (across files) and in a memory efficient way.

```
> gnCnt <- summarizeOverlaps(exByGn, sorted, "Union", TRUE,
+     singleEnd=FALSE, param=param)
```

9.4 *SummarizedExperiment*

The result of the counting exercise is a *SummarizedExperiment* object. This is an object that not only contains the matrix of counts (accessible with the `assay` function) but also the ranges on which the counts are based (`rowData`) and the phenotypic data associated with each sample. Here we explore a little:

```
> dim(gnCnt)

[1] 764   8

> hist(asinh(assay(gnCnt)))
> table(rowSums(assay(gnCnt)) == 0)

FALSE  TRUE
  514   250

> rowData(gnCnt)[[1]]      # model on which first gene counts were based

GRanges with 11 ranges and 2 metadata columns:
       seqnames               ranges strand   |   exon_id   exon_name
          <Rle>            <IRanges>  <Rle>   | <integer> <character>
   [1]    chr14 [71050957, 71051660]      -   |    184610        <NA>
   [2]    chr14 [71052473, 71052500]      -   |    184611        <NA>
   [3]    chr14 [71057983, 71058098]      -   |    184612        <NA>
   [4]    chr14 [71059228, 71059705]      -   |    184613        <NA>
   [5]    chr14 [71059295, 71060095]      -   |    184614        <NA>
   ...      ...                  ...    ... ...       ...         ...
   [7]    chr14 [71059597, 71059726]      -   |    184616        <NA>
   [8]    chr14 [71060013, 71060095]      -   |    184617        <NA>
```

[4] http://www-huber.embl.de/users/anders/HTSeq/doc/count.html

```
   [9]    chr14 [71063328, 71063419]      -  |    184618        <NA>
  [10]    chr14 [71064335, 71064494]      -  |    184619        <NA>
  [11]    chr14 [71067333, 71067384]      -  |    184620        <NA>
  ---
  seqlengths:
       chr14
   107349540
```

Here we add the phenotypic data from ArrayExpress to `gnCnt`; we want to be careful to associated the correct phenotypic data with the correct lane

```
> o <- match(pdata[["Comment.ENA_RUN."]], colnames(gnCnt))
> colData <- cbind(colData(gnCnt), as(pdata[o,], "DataFrame"))
> rownames(colData) <- rownames(colData(gnCnt))
> colData(gnCnt) <- colData
```

The merit of this structure is that we are now in a position to easily manipulate the counts without accidentally confusing row or column annotations, e.g., when selecting the 'control' samples:

```
> colData(gnCnt)$Factor.Value.RNAI.
```

```
[1] "KD1"     "KD1"     "KD2"     "KD2"     "control" "control" "control"
[8] "control"
```

```
> gnCnt[, colData(gnCnt)$Factor.Value.RNAI. == "control"]
```

```
class: SummarizedExperiment
dim: 764 4
exptData(0):
assays(1): counts
rownames(764): 10001 100113389 ... 9950 9985
rowData metadata column names(0):
colnames(4): ERR127306 ERR127307 ERR127308 ERR127309
colData names(31): fileName Source.Name ...
  Comment.READ_INDEX_1_BASE_CCORD. Factor.Value.RNAI.
```

While rigorous quality assessment should be delayed until after between-sample normalization, it is hard to resist taking a preliminary look (Figure 9.2), e.g., at the number of reads counted (compare this with the total and 'quickCountBam' reads in the file)

```
> colSums(assay(gnCnt))
```

```
ERR127302 ERR127303 ERR127304 ERR127305 ERR127306 ERR127307 ERR127308 ERR127309
   291269    311629    308206    281273    297299    321382    323936    286681
```

at the distribution of gene counts between samples and regions for the most highly expressed genes

```
> library(lattice)
> scales <- list(x=list(rot=45))
> o <- tail(order(rowMeans(assay(gnCnt))), 30)
> levelplot(asinh(t(assay(gnCnt)[o,])), scales=scales)
```

and at the distance between samples

```
> d <- dist(t(assay(gnCnt)))
> levelplot(as.matrix(d), scales=scales)
```

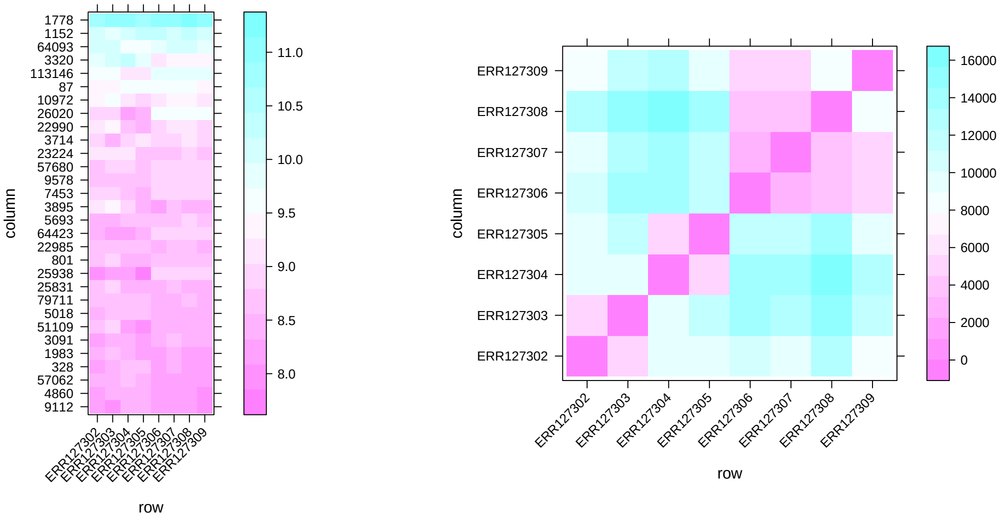


Figure 9.2: Asinh-transformed read counts amongst most strongly expressed genes (left) and Euclidean distance between samples (right) for raw data.

What patterns are suggested here? What aspects of the experimental design are these consistent with?

The `SummarizedExperiment` object can be used easily in down-stream analysis with the *edgeR* or *DESeq* packages, as illustrated on `?summarizeOverlaps`:

```
> f <- colData(gnCnt)$Factor.Value.RNAI.
> library(DESeq)
> deseq <- newCountDataSet(assays(se)$counts, f)
> library(edgeR)
> edger <- DGEList(assays(se)$counts, group=f)
```

The *DESeq2* package uses *SummarizedExperiment* objects directly.

9.5 Addiitional counting and annotation opportunities

There are additional aspects of `summarizeOverlaps` that are worth mentioning. Features can be both more and less complicated than exons grouped within genes.

9.5.1 Counting genes, counting transcripts

The `features` argument of `summarizeOverlaps` can be any `GRanges` or `GRangesList` object. As an example of a `GRanges` object, a more approximate count might tally reads overlapping the range of the exons of the gene, rather than overlapping exons. This might be appropriate if one did not have confidence in the gene model or of the sample preparation or alignment protocols. One could form the ranges in a straight-forward manner:

```
> gn <- unlist(range(exByGn))
```

For *transcript-level* differential expression, a first counting scheme is to extract exons grouped by transcript

```
> exByTx <- exonsBy(TxDb.Hsapiens.UCSC.hg19.knownGene, "tx")
> seqlevels(exByTx, force=TRUE) <- "chr14"
```

There are 2798 transcripts from the 764 genes on chromosome 14. We see that there are more counts to be estimated, and know that transcripts share greater overlap than genes. Both of these issues mean that the number of reads assigned to each transcript is less than the number of reads assigned to each gene, pointing toward the reduced power of transcript-level relative to gene-level differential expression; The *SplicingGraphs* package offers an alternative approach to describing transcripts and hence of counting reads to support transcript-level differential expression.

9.5.2 Counting by other means

There are other ways of arriving at count tables, both within and outside *Bioconductor*. The `featureCounts` function of the *Rsubread* package provides a straight-forward counting facility on Linux and MacOS, in addition to a fast aligner appropriate for use in RNA-seq differential expression [14]. The *DESeq* / *DESeq2* packages contain scripts based on HT-seq[5] python framework (no python required). Packages providing comprehensive work flows, e.g., *QuasR*, *easyRNASeq* include facilities for straight-forward counting.

9.5.3 Using other annotation resources

The `features` argument to `summarizeOverlaps` is a *GRanges* or *GRangesList* argument. These are easily created from diverse data sources. One new resource is the *AnnotationHub* *Bioconductor* package / cloud resource. The *AnnotationHub* contains *Bioconductor* representations of common resources from ensembl, UCSC, and elsewhere. Here we load the package and create a reference to the hub:

```
> library(AnnotationHub)
> hub <- AnnotationHub()
> hub
```

```
class: AnnotationHub
length: 7553
filters: none
hubUrl: http://annotationhub.bioconductor.org/ah
snapshotVersion: 2.12; snapshotDate: 2013-04-05
hubCache: /home/mtmorgan/.AnnotationHub
```

One way of exploring data on the hub is through simple tab completion, e.g.,

```
> anno <- hub$ensembl.release.70.gtf.
```

followed by the `<tab>` key provides direct access to ensembl GTF annotation files for approximately 60 organisms; these are returned to the variable `anno` as a `GRanges` object that can be easily shaped to contain desired annotations. More extensive exploration of the available data can begin by obtaining a *DataFrame* describing the resources

```
> m <- metadata(hub)
> names(m)
```

```
[1] "Description" "Genome"      "RDataClass"  "RDataPath"   "Species"
[6] "Tags"        "TaxonomyId"  "Title"
```

Other common ways of bringing resources into *Bioconductor* include the packages *rtracklayer* (for importing GTF, BED, WIG, and other common file formats) and *biomaRt* (for querying the ensembl biomart resource; these are described further in Chapters 13 and 14.

[5] http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html

Chapter 10

DESeq Work Flow Exercises

This chapter walks through an RNA-seq differential expression work flow. It is based on the *DESeq* vignette, reproduced in the Appendix A. Anders and Huber [1] provide additional information.

Exercise 12

a. *Visit the DESeq landing page*[1].

b. *Retrieve a copy of the 'Analyzing RNA-Seq data with the "DESeq" package' vignette pdf.*

c. *Retrieve a copy of the R script corresponding to the vignette.*

d. *How can the `vignette` function be used to access the vignette and script from within R, without needing to visit the Bioconductor web site?*

10.1 Data input and preparation

Exercise 13

a. *Read through sections 1 and 2 of the DESeq vignette.*

b. *Evaluate the code chunks (numbered 1 through 16) corresponding to sections 1 and 2. Step through these one at a time, reflecting on the commands that you are evaluating and the decisions that are being made about your data.*

c. *Summarize key steps that have been taken to this point, emphasizing analysis decisions you have made, the assumptions underlying these decisions, and the possible consequences of an incorrect decision.*

10.2 Inference

Exercise 14

a. *Read through section 3.1 of the DESeq vignette.*

b. *Evaluate the code chunks (17 through 28) corresponding to section 3.1.*

c. *Summarize key steps that have been taken to this point, emphasizing analysis decisions you have made, the assumptions underlying these decisions, and the possible consequences of an incorrect decision.*

[1] `http://bioconductor.org/packages/release/bioc/html/DESeq.html`

10.3 Independent filtering

Exercise 15

a. *Read through section 5 of the DESeq vignette.*

b. *Evaluate the code chunks corresponding to section 5.*

c. *How do you know when independent filtering is appropriate and effective?*

10.4 Data quality assessment

10.4.1 Preliminary transformation

Many of the data quality challenges associated with RNA-seq differential expression data are similar to those seen in microarray studies. A convenient approach to data assessment is then to transform the count data to an approximately appropriate scale, and to use transformed data and microarray quality assessment best practices to guide RNA-seq differential expression best practices. The *DESeq* vignette adopts this approach, using the variance stabilizing transformation outlined in section 6.

Exercise 16

a. *Read through the introductory part of section 6 of the DESeq vignette to gain at least a superficial understanding of the variance stabilizing transformation.*

10.4.2 Quality assessment

Exercise 17

a. *Read through section 7 of the DESeq vignette.*

b. *Evaluate the code chunks corresponding to sections 7.1 to 7.3.*

c. *Compare the two panels of the vignette Figure 15. What is the importance of data transformation prior to quality assessment?*

10.5 Frequently asked questions

No or incomplete replicates See sections 3.2 and 3.3 of the *DESeq* vignette.

Different results from different analyses Making different decisions can lead to different results. Which result is correct? The underlying decision might involve a single package (e.g., choice of dispersion estimates) or might arise from choice of packages used in an analysis (e.g., use of *DESeq* versus *edgeR*). Unfortunately, there is no straight-forward answer to which analysis is 'correct'. The appropriate analysis is the one that makes assumptions that most closely match the nature of the individual data set, or for which results are relatively robust to violations of the assumptions of the analysis.

Any rules of thumb to help identify a robust analysis?

Part III

Variant Calls

Chapter 11

Variant Work Flows

11.1 Variants

11.1.1 Varieties of variant-related work flows

- Single nucleotide polymorphism
- Copy number change
- Structural variation
- Long-range interaction

11.1.2 Work flows

- Alignment. requires tools sensitive to variation, e.g., *GSNAP*, *BWA*; *Bowtie* not optimized for this.
- Variant calling, e.g., *GATK*[1].
- Filter.
- Biological context – variant annotation.
- Integrative analysis, e.g., GWAS, genetical genomics.

11.1.3 *Bioconductor* software

Selected *Bioconductor* software relevant to DNA-seq work flows are summarized in Table 11.1. The *gmapR* package provides access to the well-respected *GSNAP* aligner. *VariantTools* is an emerging tool that works with aligned reads to call single-sample and sample-specific variants; we will work with *VariantTools* as part of this course. Packages such as *cn.mops* and *exomeCopy* identify copy number variants from high-throughput sequence data.

VariantAnnotation provides facilities for manipulated called variants stored in VCF files; the facilities are very flexible, including simple range-based filtering, look-up in dbSNP, and coding and effect prediction using standard data bases. *VariantAnnotation* plays well with *ensemblVEP* to feed data through the Ensembl Variant Effect Predictor perl script, and to *Bioconductor* facilities for SNP analysis and genetical genomics like *snpStats* and *GGtools*.

[1] `http://www.broadinstitute.org/gatk/`

Table 11.1: Selected *Bioconductor* packages for DNA-seq analysis.

Package	Description
gmapR	Alignment (Linux only)
VariantTools	Single-sample and tumor specific variant calls
deepSNV	Sub-clonal SNVs in deep sequencing experiments
cn.mops	Mixture of Poissons copy number variation estimates
exomeCopy	Hidden Markov copy number variation estimates
VariantAnnotation	Manipulating and annotating VCF files
ensemblVEP	Interface to the Ensembl Variant Effect Predictor
snpStats	Down-stream GWAS; also *GWAStools*, *GGtools*

11.2 *VariantTools*

WARNING: This portion of the course is only available on Linux. The *VariantTools* package represents a work in progress.

11.2.1 Example data: lung cancer cell lines

Exercise 18

Load the LungCancerLines experiment data package. Check out the package description (e.g., `help(package="LungCancerLines` *for a brief description of the data. Use tools from previous exercises to explore the FASTQ and BAM files that are available in this package.*

Open the VariantTools package vignette and associated script.

Solution: Here we load the package and discover paths to the BAM and FASTQ files, exploration is up to you!

```
> library(LungCancerLines)
> library(ShortRead)
> FastqFileList(LungCancerFastqFiles())
```

```
FastqFileList of length 4
names(4): H1993.first H1993.last H2073.first H2073.last
```

```
> LungCancerBamFiles()
```

```
BamFileList of length 2
names(2): H1993 H2073
```

Open the *VariantTools* vignette with

```
> vignette(package="VariantTools", "VariantTools")
```

or by visiting the package landing page[2].

11.2.2 Calling single-sample variants

Exercise 19

Create the data objects `p53`*,* `bams`*,* `bam`*, and* `tally.params` *in section 2.1 of the vignette. In particular, consult the help page* `?VariantTallyParam` *to gain insight on the parameters that you have established.*

Follow section 2.2 of the VariantTools vignette, paying particular attention to each function and the return value.

[2] http://bioconductor.org/packages/devel/bioc/html/VariantTools.html

Exercise 20

Filter variants based on standard quality filters, as in section 2.3 of the vignette.

Are there additional filters that might be appropriate at this stage of the analysis? Implement and additional filter following the hints in the brief section 2.4.

Exercise 21

Return to the bottom of page 2 of the VariantTools *package, and identify variants overlapping exons in the p53 gene.*

Exercise 22

As a simple exercise, export called variants to a VCF file following section 4 of the vignette. Spend the time to discover the relationship between the variants you have identified, their representation in R, and their representation on disk.

11.2.3 Additional work flows

Exercise 23

As time permits, explore sections of the vignette addressing variants called across samples

The previous exercise called variants in the metastatic sample, H1993. Conduct a similar analysis for the tumor sample H2073.

Explore section 3.1, calling sample-specific variants.

Chapter 12

Working with Called Variants

A major product of DNASeq experiments are catalogs of called variants (e.g., SNPs, indels). We will use the *VariantAnnotation* package to explore this type of data. Sample data included in the package are a subset of chromosome 22 from the 1000 Genomes project. Variant Call Format (VCF; full description) text files contain meta-information lines, a header line with column names, data lines with information about a position in the genome, and optional genotype information on samples for each position.

Important operations on VCF files available with the *VariantAnnotation* package are summarized in Table 12.1.

12.1 Variant call format (VCF) files with *VariantAnnotation*

12.1.1 Data input

Exercise 24
The objective of this exercise is to compare the quality of called SNPs that are located in dbSNP, versus those that are novel.

Locate the sample data in the file system. Explore the metadata (information about the content of the file) using `scanVcfHeader`*. Discover the 'info' fields* `VT` *(variant type), and* `RSQ` *(genotype imputation quality).*

Input the sample data using `readVcf`*. You'll need to specify the genome build (*`genome="hg19"`*) on which the variants are annotated. Take a peak at the* `rowData` *to see the genomic locations of each variant.*

Data resource often adopt different naming conventions for sequences. For instance, dbSNP uses abbreviations such as ch22 *to represent chromosome 22, whereas our VCF file uses 22. Use* `rowData` *and* `seqlevels<-` *to extract the row data of the variants, and rename the chromosomes.*

Solution: Explore the header:

```
> library(VariantAnnotation)
> fl <- system.file("extdata", "chr22.vcf.gz", package="VariantAnnotation")
> (hdr <- scanVcfHeader(fl))

class: VCFHeader
samples(5): HG00096 HG00097 HG00099 HG00100 HG00101
meta(1): fileformat
fixed(1): ALT
info(22): LDAF AVGPOST ... VT SNPSOURCE
geno(3): GT DS GL

> info(hdr)[c("VT", "RSQ"),]
```

Table 12.1: Working with *VCF* files and data.

Category	Function	Description
Read	`scanVcfHeader`	Retrieve file header information
	`scanVcfParam`	Select fields to read in
	`readVcf`	Read VCF file into a VCF class
	`scanVcf`	Read VCF file into a list
Filter	`filterVcf`	Filter a VCF from one file to another
Write	`writeVcf`	Write a VCF file to disk
Annotate	`locateVariants`	Identify where variant overlaps a gene annotation
	`predictCoding`	Amino acid changes for variants in coding regions
	`summarizeVariants`	Summarize variant counts by sample
SNPs	`genotypeToSnpMatrix`	Convert genotypes to a SnpMatrix
	`GLtoGP`	Convert genotype likelihoods to genotypes
	`snpSummary`	Counts and distribution statistics for SNPs
Manipulate	`expand`	Convert CompressedVCF to ExpandedVCF
	`cbind`, `rbind`	Combine by column or row

```
DataFrame with 2 rows and 3 columns
         Number        Type                                       Description
    <character> <character>                                       <character>
VT            1      String indicates what type of variant the line represents
RSQ           1       Float      Genotype imputation quality from MaCH/Thunder
```

Input the data and peak at their locations:

```
> (vcf <- readVcf(fl, "hg19"))
```

```
class: CollapsedVCF
dim: 10376 5
rowData(vcf):
  GRanges with 5 metadata columns: paramRangeID, REF, ALT, QUAL, FILTER
info(vcf):
  DataFrame with 22 columns: LDAF, AVGPOST, RSQ, ERATE, THETA, CIEND, CIPOS,...
info(header(vcf)):
             Number Type    Description
   LDAF      1      Float   MLE Allele Frequency Accounting for LD
   AVGPOST   1      Float   Average posterior probability from MaCH/Thunder
   RSQ       1      Float   Genotype imputation quality from MaCH/Thunder
   ERATE     1      Float   Per-marker Mutation rate from MaCH/Thunder
   THETA     1      Float   Per-marker Transition rate from MaCH/Thunder
   CIEND     2      Integer Confidence interval around END for imprecise va...
   CIPOS     2      Integer Confidence interval around POS for imprecise va...
   END       1      Integer End position of the variant described in this r...
   HOMLEN    .      Integer Length of base pair identical micro-homology at...
   HOMSEQ    .      String  Sequence of base pair identical micro-homology ...
   SVLEN     1      Integer Difference in length between REF and ALT alleles
   SVTYPE    1      String  Type of structural variant
   AC        .      Integer Alternate Allele Count
   AN        1      Integer Total Allele Count
   AA        1      String  Ancestral Allele, ftp://ftp.1000genomes.ebi.ac....
   AF        1      Float   Global Allele Frequency based on AC/AN
   AMR_AF    1      Float   Allele Frequency for samples from AMR based on ...
   ASN_AF    1      Float   Allele Frequency for samples from ASN based on ...
   AFR_AF    1      Float   Allele Frequency for samples from AFR based on ...
   EUR_AF    1      Float   Allele Frequency for samples from EUR based on ...
   VT        1      String  indicates what type of variant the line represents
   SNPSOURCE .      String  indicates if a snp was called when analysing th...
geno(vcf):
  SimpleList of length 3: GT, DS, GL
geno(header(vcf)):
      Number Type   Description
   GT 1      String Genotype
   DS 1      Float  Genotype dosage from MaCH/Thunder
   GL .      Float  Genotype Likelihoods
```

```
> head(rowData(vcf), 3)
```

```
GRanges with 3 ranges and 5 metadata columns:
              seqnames               ranges strand | paramRangeID
                 <Rle>            <IRanges>  <Rle> |     <factor>
    rs7410291       22 [50300078, 50300078]      * |         <NA>
  rs147922003       22 [50300086, 50300086]      * |         <NA>
  rs114143073       22 [50300101, 50300101]      * |         <NA>
                         REF                ALT      QUAL      FILTER
              <DNAStringSet> <DNAStringSetList> <numeric> <character>
    rs7410291              A                  G       100        PASS
  rs147922003              C                  T       100        PASS
  rs114143073              G                  A       100        PASS
  ---
  seqlengths:
   22
   NA
```

Rename chromosome levels:

```
> seqlevels(vcf, force=TRUE)  <- c("22"="ch22")
```

12.2 SNP Annotation

Variants can be easily identified according to region such as `coding`, `intron`, `intergenic`, `spliceSite` etc. Amino acid coding changes are computed for the non-synonymous variants. SIFT and PolyPhen databases provide predictions of how severely the coding changes affect protein function. Additional annotations are easily crafted using the *GenomicRanges* and *GenomicFeatures* software in conjunction with *Bioconductor* and broader community annotation resources.

Exercise 25
The SNPlocs.Hsapiens.dbSNP.20101109 contains information about SNPs in a particular build of dbSNP. Load the package.

Review the following short helper function to query whether SNPs are in the data base (a version of this function will be introduced to VariantAnnotation before the next release)

Figure 12.1: Quality scores of variants in dbSNP, compared to those not in dbSNP.

```
> .isInDbSNP <-
+     function(vcf, seqname, rsid=TRUE)
+ {
+     snpLocs <- getSNPlocs(seqname)
+     idx <-                     # correct seqname, width of variant == 1
+         ((seqnames(vcf) == seqname) & (width(rowData(vcf)) == 1L))
+     idx <- as.vector(idx)
+     snps <- rowData(vcf)[idx]
+     result <- rep(NA, nrow(vcf))
+     result[idx] <- if (rsid) {
+         sub("rs", "", names(snps)) %in% snpLocs[["RefSNP_id"]]
+     } else {
+         start(snps) %in% snpLocs[["loc"]]
+     }
+     result
+ }
```

Create a data frame containing the dbSNP membership status and imputation quality of each SNP. Create a density plot to illustrate the results.

Solution: Discover whether SNPs are located in dbSNP, using our helper function.

```
> library(SNPlocs.Hsapiens.dbSNP.20101109)
> inDbSNP <- .isInDbSNP(vcf, "ch22")
> table(inDbSNP)
```

Create a data frame summarizing SNP quality and dbSNP membership:

```
> metrics <-
+     data.frame(inDbSNP=inDbSNP, RSQ=info(vcf)$RSQ)
```

Finally, visualize the data, e.g., using *ggplot2* (Figure 12.1).

```
> library(ggplot2)
> ggplot(metrics, aes(RSQ, fill=inDbSNP)) +
+     geom_density(alpha=0.5) +
+     scale_x_continuous(name="MaCH / Thunder Imputation Quality") +
+     scale_y_continuous(name="Density") +
+     theme(legend.position="top")
```

Locating variants in and around genes Variant location with respect to genes can be identified with the `locateVariants` function. Regions are specified in the `region` argument and can be one of the following constructors: `CodingVariants()`, `IntronVariants()`, `FiveUTRVariants()`, `ThreeUTRVariants()`, `IntergenicVariants()`, `SpliceSiteVariants()`, or `AllVariants()`. Location definitions are shown in Table 12.2.

Exercise 26

Load the TxDb.Hsapiens.UCSC.hg19.knownGene annotation package, and read in the `chr22.vcf.gz` example file from the VariantAnnotation package.

Remembering to re-name sequence levels, use the `locateVariants` function to identify coding variants.

Summarize aspects of your data, e.g., did any coding variants match more than one gene? How many coding variants are there per gene ID?

Table 12.2: Variant locations

Location	Details
`coding`	Within a coding region
`fiveUTR`	Within a 5' untranslated region
`threeUTR`	Within a 3' untranslated region
`intron`	Within an intron region
`intergenic`	Not within a transcript associated with a gene
`spliceSite`	Overlaps any of the first or last 2 nucleotides of an intron

Solution: Here we open the known genes data base, and read in the VCF file.

```
> library(TxDb.Hsapiens.UCSC.hg19.knownGene)
> txdb <- TxDb.Hsapiens.UCSC.hg19.knownGene
> fl <- system.file("extdata", "chr22.vcf.gz", package="VariantAnnotation")
> vcf <- readVcf(fl, "hg19")
> seqlevels(vcf, force=TRUE) <- c("22"="chr22")
```

The next lines locate coding variants.

```
> rd <- rowData(vcf)
> loc <- locateVariants(rd, txdb, CodingVariants())
> head(loc, 3)
```

```
GRanges with 3 ranges and 7 metadata columns:
      seqnames               ranges strand | LOCATION   QUERYID      TXID
         <Rle>            <IRanges>  <Rle> | <factor> <integer> <integer>
  [1]    chr22 [50301422, 50301422]      * |   coding        24     73482
  [2]    chr22 [50301476, 50301476]      * |   coding        25     73482
  [3]    chr22 [50301488, 50301488]      * |   coding        26     73482
         CDSID      GENEID   PRECEDEID    FOLLOWID
      <integer> <character> <character> <character>
  [1]    217009       79087        <NA>        <NA>
  [2]    217009       79087        <NA>        <NA>
  [3]    217009       79087        <NA>        <NA>
  ---
  seqlengths:
   chr22
      NA
```

To answer gene-centric questions data can be summarized by gene regardless of transcript.

```
> ## Did any coding variants match more than one gene?
> splt <- split(loc$GENEID, loc$QUERYID)
> table(sapply(splt, function(x) length(unique(x)) > 1))
```

```
FALSE  TRUE
  956    15
```

```
> ## Summarize the number of coding variants by gene ID
> splt <- split(loc$QUERYID, loc$GENEID)
> head(sapply(splt, function(x) length(unique(x))), 3)
```

```
113730   1890  23209
    22     15     30
```

Amino acid coding changes predictCoding computes amino acid coding changes for non-synonymous variants. Only ranges in query that overlap with a coding region in subject are considered. Reference sequences are retrieved from either a BSgenome or fasta file specified in seqSource. Variant sequences are constructed by substituting, inserting or deleting values in the varAllele column into the reference sequence. Amino acid codes are computed for the variant codon sequence when the length is a multiple of 3.

The query argument to predictCoding can be a GRanges or VCF. When a GRanges is supplied the varAllele argument must be specified. In the case of a VCF object, the alternate alleles are taken from alt(<VCF>) and the varAllele argument is not specified.

The result is a modified query containing only variants that fall within coding regions. Each row represents a variant-transcript match so more than one row per original variant is possible.

```
> library(BSgenome.Hsapiens.UCSC.hg19)
> coding <- predictCoding(vcf, txdb, seqSource=Hsapiens)
> coding[5:9]
```

```
GRanges with 5 ranges and 17 metadata columns:
              seqnames               ranges strand | paramRangeID
                 <Rle>            <IRanges>  <Rle> |     <factor>
  22:50301584    chr22 [50301584, 50301584]      - |         <NA>
  rs114264124    chr22 [50302962, 50302962]      - |         <NA>
  rs149209714    chr22 [50302995, 50302995]      - |         <NA>
  22:50303554    chr22 [50303554, 50303554]      - |         <NA>
   rs12167668    chr22 [50303561, 50303561]      - |         <NA>
                           REF                ALT      QUAL      FILTER
              <DNAStringSet> <DNAStringSetList> <numeric> <character>
  22:50301584              C                  T       100        PASS
  rs114264124              C                  T       100        PASS
  rs149209714              C                  G       100        PASS
  22:50303554              T                  C       100        PASS
   rs12167668              C                  T       100        PASS
                   varAllele     CDSLOC    PROTEINLOC   QUERYID        TXID
              <DNAStringSet>  <IRanges> <IntegerList> <integer> <character>
  22:50301584              A [777, 777]           259        28       73482
  rs114264124              A [698, 698]           233        57       73482
  rs149209714              C [665, 665]           222        58       73482
  22:50303554              G [652, 652]           218        73       73482
   rs12167668              A [645, 645]           215        74       73482
                  CDSID      GENEID   CONSEQUENCE       REFCODON       VARCODON
              <integer> <character>      <factor> <DNAStringSet> <DNAStringSet>
  22:50301584    217009       79087    synonymous            CCG            CCA
  rs114264124    217010       79087 nonsynonymous            CGG            CAG
  rs149209714    217010       79087 nonsynonymous            GGA            GCA
  22:50303554    217011       79087 nonsynonymous            ATC            GTC
   rs12167668    217011       79087    synonymous            CCG            CCA
                      REFAA         VARAA
              <AAStringSet> <AAStringSet>
  22:50301584             P             P
  rs114264124             R             Q
  rs149209714             G             A
  22:50303554             I             V
   rs12167668             P             P
  ---
  seqlengths:
   chr22
      NA
```

Using variant rs114264124 as an example, we see `varAllele A` has been substituted into the `refCodon CGG` to produce `varCodon CAG`. The `refCodon` is the sequence of codons necessary to make the variant allele substitution and therefore often includes more nucleotides than indicated in the range (i.e. the range is 50302962, 50302962, width of 1). Notice it is the second position in the `refCodon` that has been substituted. This position in the codon, the position of substitution, corresponds to genomic position 50302962. This genomic position maps to position 698 in coding region-based coordinates and to triplet 233 in the protein. This is a non-synonymous coding variant where the amino acid has changed from `R` (Arg) to `Q` (Gln).

When the resulting `varCodon` is not a multiple of 3 it cannot be translated. The consequence is considered a `frameshift` and `varAA` will be missing.

```
> coding[coding$CONSEQUENCE == "frameshift"]

GRanges with 1 range and 17 metadata columns:
              seqnames               ranges strand | paramRangeID
                 <Rle>            <IRanges>  <Rle> |     <factor>
  22:50317001    chr22 [50317001, 50317001]      + |         <NA>
                         REF                ALT      QUAL      FILTER
              <DNAStringSet> <DNAStringSetList> <numeric> <character>
  22:50317001              G              GCACT       233        PASS
                   varAllele     CDSLOC    PROTEINLOC   QUERYID        TXID
              <DNAStringSet>  <IRanges> <IntegerList> <integer> <character>
  22:50317001          GCACT [808, 808]           270       359       72592
                  CDSID      GENEID CONSEQUENCE       REFCODON       VARCODON
              <integer> <character>    <factor> <DNAStringSet> <DNAStringSet>
  22:50317001    214765       79174  frameshift            GCC            GCC
                      REFAA         VARAA
              <AAStringSet> <AAStringSet>
  22:50317001             A
  ---
  seqlengths:
   chr22
      NA
```

SIFT and PolyPhen databases From `predictCoding` we identified the amino acid coding changes for the non-synonymous variants. For this subset we can retrieve predictions of how damaging these coding changes may be. SIFT (Sorting Intolerant From Tolerant) and PolyPhen (Polymorphism Phenotyping) are methods that predict the impact of amino acid substitution on a human protein. The SIFT method uses sequence homology and the physical properties of amino acids to make predictions about protein function. PolyPhen uses sequence-based features and structural information characterizing the substitution to make predictions about the structure and function of the protein.

Collated predictions for specific dbSNP builds are available as downloads from the SIFT and PolyPhen web sites. These results have been packaged into *SIFT.Hsapiens.dbSNP132.db* and *PolyPhen.Hapiens.dbSNP131.db* and are designed to be searched by rsid. Variants that are in dbSNP can be searched with these database packages. When working with novel variants, SIFT and PolyPhen must be called directly. See references for home pages.

Identify the non-synonymous variants and obtain the rsids.

```
> nms <- names(coding)
> idx <- coding$CONSEQUENCE == "nonsynonymous"
> nonsyn <- coding[idx]
> names(nonsyn) <- nms[idx]
> rsids <- unique(names(nonsyn)[grep("rs", names(nonsyn), fixed=TRUE)])
```

Detailed descriptions of the database columns can be found with `?SIFTDbColumns` and `?PolyPhenDbColumns`. Variants in these databases often contain more than one row per variant. The variant may have been reported by multiple sources and therefore the source will differ as well as some of the other variables.

```
> library(SIFT.Hsapiens.dbSNP132)
> ## rsids in the package
> head(keys(SIFT.Hsapiens.dbSNP132), 3)

[1] "rs47"  "rs268" "rs298"

> ## list available columns
> cols(SIFT.Hsapiens.dbSNP132)

 [1] "RSID"         "PROTEINID"    "AACHANGE"     "METHOD"       "AA"
 [6] "PREDICTION"   "SCORE"        "MEDIAN"       "POSTIONSEQS" "TOTALSEQS"

> ## select a subset of columns
> ## a warning is thrown when a key is not found in the database
> subst <- c("RSID", "PREDICTION", "SCORE", "AACHANGE", "PROTEINID")
> sift <- select(SIFT.Hsapiens.dbSNP132, keys=rsids, cols=subst)
> head(sift, 3)

         RSID PROTEINID AACHANGE PREDICTION SCORE
1 rs114264124 NP_077010    R233Q  TOLERATED  0.59
2 rs114264124 NP_077010    R233Q  TOLERATED  1.00
3 rs114264124 NP_077010    R233Q  TOLERATED  0.20
```

PolyPhen provides predictions using two different training datasets and has considerable information about 3D protein structure. See `?PolyPhenDbColumns` or the PolyPhen web site listed in the references for more details.

12.3 Annotation with *ensemblVEP*

```
> library(ensemblVEP)
```

12.4 Large-scale filtering

One source of VCF files is from whole-genome sequencing and variant calling; an example data set is from Complete Genomics, and a subset is available on the Amazon Machine instance accompanying this course

```
> vcfDir <- "~/Seattle-May-2013/CompleteGenomics"
> dir(vcfDir)
> vcfFile <- dir(vcfDir, ".gz$", full=TRUE)
```

The data were retrieved from the Complete Genomics web site, the first 750,000 of about 14 million variants selected, and the resulting file compressed and indexed. Indexing makes them accessible to fast queries using `which` argument to `ScanVcfParam`.

Exercise 27
The objective of this exercise is to filter the larger VCF file to a subset of interesting variants that we might wish to study in depth at a later date. We use the `filterVcf` *function of the* VariantAnnotation *package to perform the filtering.*

Start by taking a look at the (complicated) header information

```
> hdr <- scanVcfHeader(vcfFile)
```

We'll be paying attention to the `SS` *INFO field, and the* `AD` *GENO field. Determine the data types and possible values for these fields, using commands like*

```
> info(hdr)
> geno(hdr)
```

What data type would you use to represent the `SS` *field for a single or several VCF records in R? What about the* `AD` *field, across all samples?*

This VCF file is big. While we can read this into memory all at once, we will often want to 'chunk' through a file, reading many (e.g., a million) records at a time. We do this by creating a TabixFile and specifying a `yieldSize` *representing the size of the file that we'd like to read at each go. Create a TabixFile with* `yieldSize=100000` *and verify with a simple loop that the entire file appears to be processed in chunks of the specified size, along the lines of. . .*

```
> tbx <- TabixFile(vcfFile, yieldSize=100000)
> open(tbx)
> while (len <- nrow(readVcf(tbx, "hg19")))
+     cat("read", len, "rows\n")
```

As you can see from the chunking exercise, it takes quite a bit of time to process these lines. To filter 14 million variants effectively, it can pay to do a cheaper 'pre-filter'. Specifically, we're interested in variants tagged 'Germline'. If we were to represents each VCF record as an element of a character vector `x`*, then we could write a one-liner that returned* `TRUE` *if the line contained the word 'Germline':*

```
> grepl("Germline", x, fixed=TRUE)
```

This would be fast to read in to R, and fast to perform the filter. The `filterVcf` *function allows us to specify a pre-filter that works just like this. The filters are constructed using the FilterRules constructor in* IRanges*, by translating our one-liner into a simple function call, and placing the function call into a list.*

```
> isGermline <- function(x)
+     grepl("Germline", x, fixed=TRUE)
> filters <- FilterRules(list(isGermline=isGermline))
```

The idea is that several filters are chained together. Each filter returns a logical vector indicating the subset of data to be processed by the next filter.

Here is our pre-filter in action:

```
> destination <- tempfile()               # temporary location
> filterVcf(vcfFile, "hg19", destination, prefilters=filters)
```

This is pretty fast, and drops the number of variants under consideration quite substantially, to about 110000.

Our next filter is more challenging to write. We're interested in allelic depth, a summary of the evidence for the variant summarized in the `AD` *GENO field. There are many variants, each sample has two values of AD, and there are two samples. This means that* `AD` *is a three-dimensional array. Our filter criteria is that the ratio of ('alternate allele' of the tumor sample* or *the 'reference allele' of the the normal sample) to total reads is greater than 0.1. Here's function implementing this:*

```
> allelicDepth <- function(x)
+ {
+     ##  ratio of AD of the 'alternate allele' for the tumor sample
+     ##  OR 'reference allele' for normal samples to total reads for
+     ##  the sample should be greater than some threshold (say 0.1,
+     ##  that is: at least 10% of the sample should have the allele
+     ##  of interest)
+     ad <- geno(x)[["AD"]]
+     tumorPct <- ad[,1,2,drop=FALSE] / rowSums(ad[,1,,drop=FALSE])
+     normPct <- ad[,2,1, drop=FALSE] / rowSums(ad[,2,,drop=FALSE])
+     test <- (tumorPct > 0.1) | (normPct > 0.1)
+     !is.na(test) & test
+ }
```

We can add it to our list of filters

```
> filters <- FilterRules(list(isGermline=isGermline,
+                             allelicDepth=allelicDepth))
```

To use this filter, we actually need to fully parse the VCF file into the VCF instance; the pre-filter trick used for germline filtering is not enough. `filterVcf` allows us to perform a filter on the VCF instance, too, and does so after pre-filtering

```
> destination <- tempfile()
> filterVcf(vcfFile, "hg19", destination, prefilters=filters[1],
+           filters=filters[2])
```

And finally input our interesting variants, confirming that we've done our filtering as desired.

```
> vcf <- readVcf(destination, "hg19")
> all(info(vcf)$SS == "Germline")
> table(allelicDepth(vcf))
```

Part IV

Annotation and Visualization

Chapter 13

Gene-centric Annotation

Bioconductor provides extensive annotation resources, summarized in Figure 13.1. These can be *gene*-, or *genome*-centric. Annotations can be provided in packages curated by *Bioconductor*, or obtained from web-based resources. Gene-centric *AnnotationDbi* packages include:

- Organism level: e.g. *org.Mm.eg.db*, *Homo.sapiens*.
- Platform level: e.g. *hgu133plus2.db*, *hgu133plus2.probes*, *hgu133plus2.cdf*.
- Homology level: e.g. *hom.Dm.inp.db*.
- System biology level: *GO.db*, *KEGG.db*, *Reactome.db*.

Examples of genome-centric packages include:

- *GenomicFeatures*, to represent genomic features, including constructing reproducible feature or transcript data bases from file or web resources.
- Pre-built transcriptome packages, e.g. *TxDb.Hsapiens.UCSC.hg19.knownGene* based on the *H. sapiens* UCSC hg19 knownGenes track.
- *BSgenome* for whole genome sequence representation and manipulation.
- Pre-built genomes, e.g., *BSgenome.Hsapiens.UCSC.hg19* based on the *H. sapiens* UCSC hg19 build.

Web-based resources include

- *biomaRt* to query biomart resource for genes, sequence, SNPs, and etc.
- *rtracklayer* for interfacing with browser tracks, especially the UCSC genome browser.

13.1 Gene-centric annotations with *AnnotationDbi*

Organism-level ('org') packages contain mappings between a central identifier (e.g., Entrez gene ids) and other identifiers (e.g. GenBank or Uniprot accession number, RefSeq id, etc.). The name of an org package is always of the form *org.<Sp>.<id>.db* (e.g. *org.Sc.sgd.db*) where *<Sp>* is a 2-letter abbreviation of the organism (e.g. `Sc` for *Saccharomyces cerevisiae*) and *<id>* is an abbreviation (in lower-case) describing the type of central identifier (e.g. `sgd` for gene identifiers assigned by the *Saccharomyces* Genome Database, or `eg` for Entrez gene ids). The "How to use the '.db' annotation packages" vignette in the *AnnotationDbi* package (org packages are only one type of ".db" annotation packages) is a key reference. The '.db' and most other *Bioconductor* annotation packages are updated every 6 months.

Annotation packages contain an object named after the package itself. These objects are collectively called *AnnotationDb* objects, with more specific classes named *OrgDb*, *ChipDb* or *TranscriptDb* objects. Methods that can be applied to these objects include `cols`, `keys`, `keytypes` and `select`. Common operations for retrieving annotations are summarized in Table 13.1.

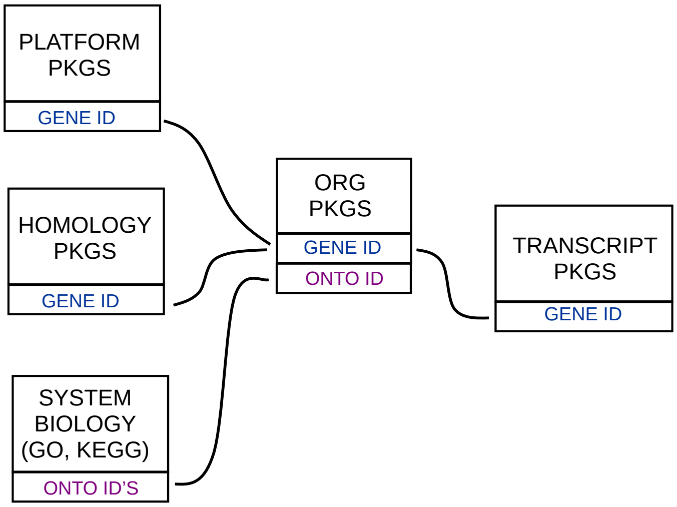


Figure 13.1: Annotation Packages: the big picture

Table 13.1: Common operations for retrieving and manipulating annotations.

Category	Function	Description
Discover	`cols`	List the kinds of columns that can be returned
	`keytypes`	List columns that can be used as keys
	`keys`	List values that can be expected for a given keytype
	`select`	Retrieve annotations matching `keys`, `keytype` and `cols`
Manipulate	`setdiff`, `union`, `intersect`	Operations on sets
	`duplicated`, `unique`	Mark or remove duplicates
	`%in%`, `match`	Find matches
	`any`, `all`	Are any `TRUE`? Are all?
	`merge`	Combine two different `data.frames` based on shared keys
*GRanges**	`transcripts`, `exons`, `cds`	Features (transcripts, exons, coding sequence) as *GRanges*.
	`transcriptsBy` , `exonsBy`	Features group by gene, transcript, etc., as *GRangesList*.
	`cdsBy`	

Exercise 28
What is the name of the org package for Drosophila*? Load it. Display the OrgDb object for the* org.Dm.eg.db *package. Use the* `cols` *method to discover which sorts of annotations can be extracted from it.*

Use the `keys` *method to extract UNIPROT identifiers and then pass those keys in to the* `select` *method in such a way that you extract the SYMBOL (gene symbol) and KEGG pathway information for each.*

Use `select` *to retrieve the ENTREZ and SYMBOL identifiers of all genes in the KEGG pathway* `00310`*.*

Solution: The *OrgDb* object is named `org.Dm.eg.db`.

```
> library(org.Dm.eg.db)
> cols(org.Dm.eg.db)

 [1] "ENTREZID"     "ACCNUM"       "ALIAS"        "CHR"          "CHRLOC"
 [6] "CHRLOCEND"    "ENZYME"       "MAP"          "PATH"         "PMID"
[11] "REFSEQ"       "SYMBOL"       "UNIGENE"      "ENSEMBL"      "ENSEMBLPROT"
[16] "ENSEMBLTRANS" "GENENAME"     "UNIPROT"      "GO"           "EVIDENCE"
[21] "ONTOLOGY"     "GOALL"        "EVIDENCEALL"  "ONTOLOGYALL"  "FLYBASE"
[26] "FLYBASECG"    "FLYBASEPROT"

> keytypes(org.Dm.eg.db)

 [1] "ENTREZID"     "ACCNUM"       "ALIAS"        "CHR"          "CHRLOC"
 [6] "CHRLOCEND"    "ENZYME"       "MAP"          "PATH"         "PMID"
[11] "REFSEQ"       "SYMBOL"       "UNIGENE"      "ENSEMBL"      "ENSEMBLPROT"
[16] "ENSEMBLTRANS" "GENENAME"     "UNIPROT"      "GO"           "EVIDENCE"
[21] "ONTOLOGY"     "GOALL"        "EVIDENCEALL"  "ONTOLOGYALL"  "FLYBASE"
[26] "FLYBASECG"    "FLYBASEPROT"

> uniprotKeys <- head(keys(org.Dm.eg.db, keytype="UNIPROT"))
> cols <- c("SYMBOL", "PATH")
> select(org.Dm.eg.db, keys=uniprotKeys, cols=cols, keytype="UNIPROT")

  UNIPROT  SYMBOL  PATH
1  Q8IRZ0  CG3038  <NA>
2  Q95RP8  CG3038  <NA>
3  Q95RU8     G9a 00310
4  Q9W5H1 CG13377  <NA>
5  P39205     cin  <NA>
6  Q24312     ewg  <NA>
```

Selecting UNIPROT and SYMBOL ids of KEGG pathway `00310` is very similar:

```
> kegg <- select(org.Dm.eg.db, "00310", c("UNIPROT", "SYMBOL"), "PATH")
> nrow(kegg)

[1] 32

> head(kegg, 3)

   PATH UNIPROT  SYMBOL
1 00310  Q95RU8     G9a
2 00310  Q9W5E0 Hmt4-20
3 00310  Q9W3N9 CG10932
```

Table 13.2: Selected packages querying web-based annotation services.

Package	Description
AnnotationHub	Ensembl, Encode, dbSNP, UCSC data objects
biomaRt	`http://biomart.org`, Ensembl and other annotations
uniprot.ws	`http://uniprot.org`, protein annotations
KEGGREST	`http://www.genome.jp/kegg`, KEGG pathways
SRAdb	`http://www.ncbi.nlm.nih.gov/sra`, sequencing experiments.
rtracklayer	`http://genome.ucsc.edu`, genome tracks.
GEOquery	`http://www.ncbi.nlm.nih.gov/geo/`, array and other data
ArrayExpress	`http://www.ebi.ac.uk/arrayexpress/`, array and other data

Exercise 29
For convenience, `lrTest`, a DGEGLM object from the RNA-seq chapter, is included in the SequenceAnalysisData package. The following code loads this data and creates a 'top table' of the ten most differentially represented genes. This top table is then coerced to a `data.frame`.

```
> library(SequenceAnalysisData)
> library(edgeR)
> library(org.Dm.eg.db)
> data(lrTest)
> tt <- as.data.frame(topTags(lrTest))
```

Extract the Flybase gene identifiers (`FLYBASE`) from the row names of this table and map them to their corresponding Entrez gene (`ENTREZID`) and symbol ids (`SYMBOL`) using `select`. Use `merge` to add the results of `select` to the top table.

Solution:

```
> fbids <- rownames(tt)
> cols <- c("ENTREZID", "SYMBOL")
> anno <- select(org.Dm.eg.db, fbids, cols, "FLYBASE")
> ttanno <- merge(tt, anno, by.x=0, by.y="FLYBASE")
> dim(ttanno)
```

```
[1] 10  8
```

```
> head(ttanno, 3)
```

```
    Row.names logConc logFC LR.statistic  PValue     FDR ENTREZID  SYMBOL
1 FBgn0000071     -11   2.8          183 1.1e-41 1.1e-38    40831     Ama
2 FBgn0024288     -12  -4.7          179 7.1e-41 6.3e-38    45039 Sox100B
3 FBgn0033764     -12   3.5          188 6.8e-43 7.8e-40     <NA>    <NA>
```

13.2 *biomaRt* and other web-based resources

A short summary of select *Bioconductor* packages enabling web-based queries is in Table 13.2.

13.2.1 Using biomaRt

The *biomaRt* package offers access to the online biomart resource. this consists of several data base resources, referred to as 'marts'. Each mart allows access to multiple data sets; the *biomaRt* package provides methods for mart and data set discovery, and a standard method `getBM` to retrieve data.

Exercise 30
Load the biomaRt *package and list the available marts. Choose the* ensembl *mart and list the datasets for that mart. Set up a mart to use the* ensembl *mart and the* hsapiens_gene_ensembl *dataset.*

A biomaRt *dataset can be accessed via* `getBM`*. In addition to the mart to be accessed, this function takes filters and attributes as arguments. Use* `filterOptions` *and* `listAttributes` *to discover values for these arguments. Call* `getBM` *using filters and attributes of your choosing.*

Solution:

```
> library(biomaRt)
> head(listMarts(), 3)                      ## list the marts
> head(listDatasets(useMart("ensembl")), 3) ## mart datasets
> ensembl <-                                ## fully specified mart
+     useMart("ensembl", dataset = "hsapiens_gene_ensembl")
> head(listFilters(ensembl), 3)             ## filters
> myFilter <- "chromosome_name"
> head(filterOptions(myFilter, ensembl), 3) ## return values
> myValues <- c("21", "22")
> head(listAttributes(ensembl), 3)          ## attributes
> myAttributes <- c("ensembl_gene_id","chromosome_name")
> ## assemble and query the mart
> res <- getBM(attributes =  myAttributes, filters =  myFilter,
+              values =  myValues, mart = ensembl)
```

Use `head(res)` to see the results.

Chapter 14

Genomic Annotation

14.1 *AnnotationHub*

14.2 Whole genome sequences

There are a diversity of packages and classes available for representing large genomes. Several include:

TxDb.* For transcript and other genome / coordinate annotation.

BSgenome For whole-genome representation. See `available.packages` for pre-packaged genomes, and the vignette 'How to forge a BSgenome data package' in the

Homo.sapiens For integrating *TxDb** and *org.** packages.

SNPlocs.* For model organism SNP locations derived from dbSNP.

`FaFile` (*Rsamtools*) for accessing indexed FASTA files.

SIFT.*, ***PolyPhen*** Variant effect scores.

14.3 Gene models

14.3.1 *TxDb.** packages for model organisms

Genome-centric packages are very useful for annotations involving genomic coordinates. It is straight-forward, for instance, to discover the coordinates of coding sequences in regions of interest, and from these retrieve corresponding DNA or protein coding sequences. Other examples of the types of operations that are easy to perform with genome-centric annotations include defining regions of interest for counting aligned reads

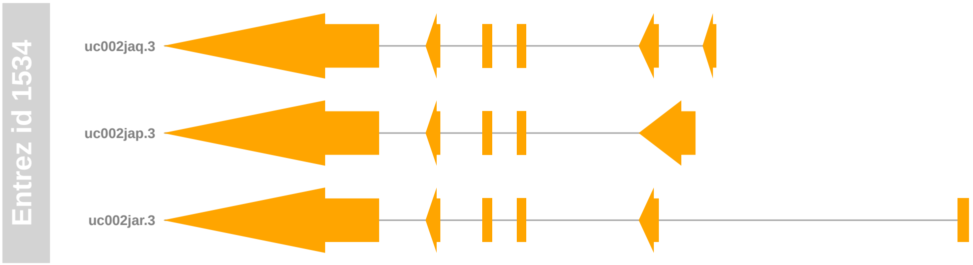


Figure 14.1: Gene model showing exons on three transcripts.

in RNA-seq experiments and retrieving DNA sequences underlying regions of interest in ChIP-seq analysis, e.g., for motif characterization.

Exercise 31
Load the 'transcript.db' package relevant to the dm3 build of D. melanogaster. *Use* `select` *and friends to select the Flybase gene ids of the top table* `tt` *and the Flybase transcript names (TXNAME) and Entrez gene identifiers (GENEID).*

Use `cdsBy` *to extract all coding sequences, grouped by transcript. Subset the coding sequences to contain just the transcripts relevant to the top table. How many transcripts are there? What is the structure of the first transcript's coding sequence?*

Load the 'BSgenome' package for the dm3 build of D. melanogaster. *Use the coding sequences ranges of the previous part of this exercise to extract the underlying DNA sequence, using the* `extractTranscriptsFromGenome` *function. Use Biostrings'* `translate` *to convert DNA to amino acid sequences.*

Solution: The following loads the relevant Transcript.db package, and creates a more convenient alias to the *TranscriptDb* instance defined in the package.

```
> library(TxDb.Dmelanogaster.UCSC.dm3.ensGene)
> txdb <- TxDb.Dmelanogaster.UCSC.dm3.ensGene
```

We also need the data – flybase IDs from our differential expression analysis.

```
> library(SequenceAnalysisData)
> data(lrTest)
> fbids <- rownames(topTags(lrTest))
```

We can discover available keys (using `keys`) and columns (`cols`) in `txdb`, and then use `select` to retrieve the transcripts associated with each differentially expressed gene. The mapping between gene and transcript is not one-to-one – some genes have more than one transcript.

```
> txnm <- select(txdb, fbids, "TXNAME", "GENEID")
> nrow(txnm)

[1] 19

> head(txnm, 3)

       GENEID      TXNAME
1 FBgn0039155 FBtr0084549
2 FBgn0039827 FBtr0085755
3 FBgn0039827 FBtr0085756
```

The *TranscriptDb* instances can be queried for data that is more structured than simple data frames, and in particular return *GRanges* or *GRangesList* instances to represent genomic coordinates. These queries are performed using `cdsBy` (coding sequence), `transcriptsBy` (transcripts), etc., where a function argument `by` specifies how coding sequences or transcripts are grouped. Here we extract the coding sequences grouped by transcript, returning the transcript names, and subset the resulting *GRangesList* to contain just the transcripts of interest to us. The first transcript is composed of 6 distinct coding sequence regions.

```
> cds <- cdsBy(txdb, "tx", use.names=TRUE)[txnm$TXNAME]
> length(cds)

[1] 19

> cds[1]
```

```
GRangesList of length 1:
$FBtr0084549
GRanges with 6 ranges and 3 metadata columns:
      seqnames               ranges strand |    cds_id    cds_name exon_rank
         <Rle>            <IRanges>  <Rle> | <integer> <character> <integer>
  [1]    chr3R [19970946, 19971592]      + |     39378        <NA>         2
  [2]    chr3R [19971652, 19971770]      + |     39379        <NA>         3
  [3]    chr3R [19971831, 19972024]      + |     39380        <NA>         4
  [4]    chr3R [19972088, 19972461]      + |     39381        <NA>         5
  [5]    chr3R [19972523, 19972589]      + |     39382        <NA>         6
  [6]    chr3R [19972918, 19973094]      + |     39383        <NA>         7

---
seqlengths:
     chr2L     chr2R     chr3L     chr3R ...   chrXHet   chrYHet chrUextra
  23011544  21146708  24543557  27905053 ...    204112    347038  29004656
```

The following code loads the appropriate BSgenome package; the `Dmelanogaster` object refers to the whole genome sequence represented in this package. The remaining steps extract the DNA sequence of each transcript, and translates these to amino acid sequences. Issues of strand are handled correctly.

```
> library(BSgenome.Dmelanogaster.UCSC.dm3)
> txx <- extractTranscriptsFromGenome(Dmelanogaster, cds)
> length(txx)
```

```
[1] 19
```

```
> head(txx, 3)
```

```
  A DNAStringSet instance of length 3
    width seq                                               names
[1]  1578 ATGGGCAGCATGCAAGTGGCGCT...TGCAGATCAAGTGCAGCGACTAG FBtr0084549
[2]  2760 ATGCTGCGTTATCTGGCGCTTTC...TTGCTGCCCCATTCGAACTTTAG FBtr0085755
[3]  2217 ATGGCACTCAAGTTTCCCACAGT...TTGCTGCCCCATTCGAACTTTAG FBtr0085756
```

```
> head(translate(txx), 3)
```

```
  A AAStringSet instance of length 3
    width seq
[1]   526 MGSMQVALLALLVLGQLFPSAVANGSSSYSSTST...VLDDSRNVFTFTTPKCENFRKRFPKLQIKCSD*
[2]   920 MLRYLALSEAGIAKLPRPQSRCYHSEKGVWGYKP...YCGRCEAPTPATGIGKVHKREVDEIVAAPFEL*
[3]   739 MALKFPTVKRYGGEGAESMLAFFWQLLRDSVQAN...YCGRCEAPTPATGIGKVHKREVDEIVAAPFEL*
```

14.4 UCSC tracks

14.4.1 Easily creating *TranscriptDb* objects from GTF files

Exercise 32
(Adapted from[1]) The 'track' curated annotations at UCSC are a great resource; this exercise creates a TranscriptDb instance from one such track.

[1] http://www.sph.emory.edu/~hwu/teaching/bioc/bios560R.html

a. *Load the GenomicFeatures and rtracklayer packages.*

b. *Discover available genomes with* `ucscGenomes`*, and available tables with the* `supportedUCSCtables`*.*

c. *Use the* `makeTranscriptDbFromUCSC` *from a suitable track, e.g., genome* `genome="ce2", tablename="refGene"`*. There are some warnings; are these something to be concerned about?*

d. *Exercise the object that you've created, e.g., exploring the basis of the warnings.*

e. *Save and load the TranscriptDb object you've created, illustrating how one can make these annotations convenient and reproducible.*

f. *What's the difference between* `makeTranscriptDbFromUCSC` *and* `makeFeatureDbFromUCSC`*? Where else can transcript and feature data bases be made from?*

Solution: Load the *GenomicFeatures* package and discover available genomes and tables:

```
> library(rtracklayer)
> library(GenomicFeatures)
> ## genomes
> gnms <- ucscGenomes()
> nrow(gnms)
```

```
[1] 146
```

```
> gnms[grep("elegans", gnms$species),]
```

```
      db    species      date              name
135 ce10 C. elegans Oct. 2010 WormBase v. WS220
136  ce6 C. elegans  May 2008 WormBase v. WS190
137  ce4 C. elegans Jan. 2007 WormBase v. WS170
138  ce2 C. elegans Mar. 2004 WormBase v. WS120
```

```
> ## tables
> tbls <- supportedUCSCtables()
> nrow(tbls)
```

```
[1] 25
```

```
> head(tbls)
```

```
                                 track        subtrack
knownGene                   UCSC Genes            <NA>
knownGeneOld3           Old UCSC Genes            <NA>
wgEncodeGencodeManualV3  Gencode Genes Genecode Manual
wgEncodeGencodeAutoV3    Gencode Genes   Genecode Auto
wgEncodeGencodePolyaV3   Gencode Genes  Genecode PolyA
ccdsGene                          CCDS            <NA>
```

Make the *TranscriptDb* object (this will take a minute)

```
> ## Not run
> txdb <- makeTranscriptDbFromUCSC("ce10", "refGene")
> saveDb(txdb, file="/path/to/file.sqlite")
```

The warnings during object creation are about unusual lengths for CDS (coding sequences should be in multiples of 3, since there are three nucleotide residues per amino acid residue).

```
1: In .extractUCSCCdsStartEnd(cdsStart[i], cdsEnd[i], ... :
  UCSC data anomaly in transcript NM_001129046: the cds cumulative
  length is not a multiple of 3
```

but we seem to have a useful object with relevant metadata information for reproducible research:

```
> txdb
```

```
TranscriptDb object:
| Db type: TranscriptDb
| Supporting package: GenomicFeatures
| Data source: UCSC
| Genome: ce10
| Organism: Caenorhabditis elegans
| UCSC Table: refGene
| Resource URL: http://genome.ucsc.edu/
| Type of Gene ID: Entrez Gene ID
| Full dataset: yes
| miRBase build ID: NA
| transcript_nrow: 48714
| exon_nrow: 152542
| cds_nrow: 129947
| Db created by: GenomicFeatures package from Bioconductor
| Creation time: 2013-02-10 10:33:50 -0800 (Sun, 10 Feb 2013)
| GenomicFeatures version at creation time: 1.11.8
| RSQLite version at creation time: 0.11.2
| DBSCHEMAVERSION: 1.0
```

Let's investigate the source of the warnings – what fraction of the CDS have lengths that are not a multiple of 3? To do this we'll need to assemble the figure out the sum of the widths of the coding sequence exons in each transcript. Start by extracting all coding sequence exons, grouped by transcript; verify that this is a *GRangesList* with a reasonable number of entries.

```
> cdsByTx <- cdsBy(txdb, "tx", use.names=TRUE)
> length(cdsByTx)
```

```
[1] 26146
```

```
> cdsByTx[1:2]
```

```
GRangesList of length 2:
$NM_058259
GRanges with 3 ranges and 3 metadata columns:
      seqnames         ranges strand |    cds_id    cds_name exon_rank
         <Rle>      <IRanges>  <Rle> | <integer> <character> <integer>
  [1]     chrI [11641, 11689]      + |         1        <NA>         1
  [2]     chrI [14951, 15160]      + |         2        <NA>         2
  [3]     chrI [16473, 16585]      + |         3        <NA>         3

$NM_058264
GRanges with 5 ranges and 3 metadata columns:
      seqnames         ranges strand | cds_id cds_name exon_rank
  [1]     chrI [43733, 43961]      + |      4     <NA>         1
  [2]     chrI [44030, 44234]      + |      5     <NA>         2
  [3]     chrI [44281, 44324]      + |       6     <NA>         3
  [4]     chrI [44372, 44468]      + |       7     <NA>         4
  [5]     chrI [44521, 44677]      + |       8     <NA>         5

---
seqlengths:
     chrI    chrII   chrIII    chrIV     chrV     chrX     chrM
 15072423 15279345 13783700 17493793 20924149 17718866    13794
```

Extract the width of each each exon; this is an *IntegerList* instance with one element of the list for each transcript, and one integer value for each exon.

```
> wd <- width(cdsByTx)
> length(wd)

[1] 26146

> head(wd, 3)

CompressedIntegerList of length 3
[["NM_058259"]] 49 210 113
[["NM_058264"]] 229 205 44 97 157
[["NM_001026606"]] 139 163 183 166
```

Use `sum` to add up the widths within each list element; note that we're using the `sum,CompressedIntegerList-method`, and that this has been specialized to do the summation within list elements.

```
> head(sum(wd))

   NM_058259    NM_058264 NM_001026606    NM_058265 NM_001026607    NM_182094
         372          732          651         1341         1620         1221
```

We now have a standard *R* vector; a one-liner asking about the number of transcripts with exons that do not sum to 3 is

```
> table( (sum(width(cdsBy(txdb, "tx"))) %% 3) != 0 )

FALSE  TRUE
25676   470
```

Exercise 33

(Adapted from[2]) Suppose you have a list of transcription factor binding sites on hg19. How would you obtain (a) the GC content of each site and (b) the percentage of gene promoters covered by the binding sites?

Solution: As an outline of a solution, the steps for calculating GC content might be

a. Represent the list of transcription factor binding sites ('regions of interest') as a *GRanges* instance, `roi`.
b. Load *BSgenome* and the appropriate genome package, e.g., *BSgenome.Hsapiens.UCSC.hg19*.
c. Use `getSeq` to retrieve the sequences, `seqs <- getSeq(Hsapiens, gr)`.
d. Use `alphabetFrequency(seqs)` to summarize nucleotide use, and simple *R* functions to determine GC content of each region of interest.

[2] http://www.sph.emory.edu/~hwu/teaching/bioc/bios560R.html

e. Summarize these as density plots, etc. A meaningful extension of this exercise might compare the observed GC content to the expected content, where expectation is the product of the independent G and C frequencies.

To calculate the percentage of promoters covered by binding sites, we might

a. Load the reference genome *TxDb* package, *TxDb.Hsapiens.UCSC.hg19.knownGene*.

b. Query the package for promoters using the `promoters` function, or otherwise manipulating exon or transcript coordinates to get a *GRanges* or *GRangesList* representing genomic regions of interest, `groi`.

c. Use `countOverlaps(groi, roi)` to find how many transcription factor binding sites overlap each promoter, and from there use standard *R* functions to tally the number of promoters that have zero overlaps.

Chapter 15

Visualizing Sequence Data

R has some great visualization packages; essential references include [6] for a general introduction, Murrell [19] for base graphics, Sarkar [25] for *lattice*, and Wickham [27] for *ggplot2*. Here we take a quick tour of visualization facilities tailed for sequence data and using *Bioconductor* approaches.

15.1 *Gviz*

The *Gviz* package produces very elegant data organized in a more-or-less familiar 'track' format. The following exercises walk through the *Gviz* User guide Section 2.

Exercise 34
Load the Gviz package and sample GRanges containing genomic coordinates of CpG islands. Create a couple of variables with information on the chromosome and genome of the data (how can this information be extracted from the `cpgIslands` *object?).*

```
> library(Gviz)
> data(cpgIslands)
> chr <- "chr7"
> genome <- "hg19"
```

The basic idea is to create a track, perhaps with additional attributes, and to plot it. There are different types of track, and we create these one at a time. We start with a simple annotation track

```
> atrack <- AnnotationTrack(cpgIslands, name="CpG")
> plotTracks(atrack)
```

Then add a track that represents genomic coordinates. Tracks are combined during when plotted, as a simple list. The vertical ordering of tracks is determined by their position in the list.

```
> gtrack <- GenomeAxisTrack()
> plotTracks(list(gtrack, atrack))
```

We can add an ideogram to provide overall orientation…

```
> itrack <- IdeogramTrack(genome=genome, chromosome=chr)
> plotTracks(list(itrack, gtrack, atrack))
```

and a more elaborate gene model, as an *data.frame* or *GRanges* object with specific columns of metadata.

```
> data(geneModels)
> grtrack <-
+     GeneRegionTrack(geneModels, genome=genome,
+                     chromosome=chr, name="Gene Model")
> tracks <- list(itrack, gtrack, atrack, grtrack)
> plotTracks(tracks)
```

Zooming out changes the location box on the ideogram

```
> plotTracks(tracks, from=2.5e7, to=2.8e7)
```

When zoomed in we can add sequence data

```
> library(BSgenome.Hsapiens.UCSC.hg19)
> strack <- SequenceTrack(Hsapiens, chromosome=chr)
> plotTracks(c(tracks, strack), from=26450430, to=26450490, cex=.8)
```

As the *Gviz* vignette humbly says, 'so far we have replicated the features of a whole bunch of other genome browser tools out there'. We'd like to be able integrate our data into these plots, with a rich range of plotting options. The key is the `DataTrack` function, which we demonstrate with some simulated data; this final result is shown in Figure 15.1.

```
> ## some data
> lim <- c(26700000, 26900000)
> coords <- seq(lim[1], lim[2], 101)
> dat <- runif(length(coords) - 1, min=-10, max=10)
> ## DataTrack
> dtrack <-
+     DataTrack(data=dat, start=coords[-length(coords)],
+               end= coords[-1], chromosome=chr, genome=genome,
+               name="Uniform Random")
> plotTracks(c(tracks, dtrack))
```

Section 4.3 of the *Gviz* vignette illustrates flexibility of the data track.

15.2 *ggbio*

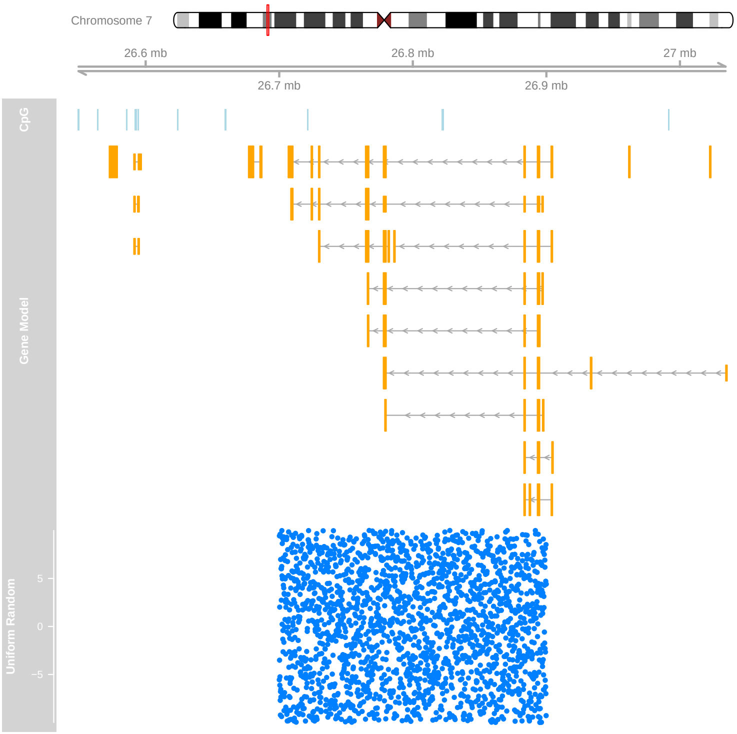
The *ggbio* package complements facilities in *Gviz*. It uses the central metaphors of the 'grammar of graphics' made popular by the *ggplot2* package, and integrates closely with the *Bioconductor* ranges infrastructure. In addition to the grammar of graphics approach, the package offers a wider range of plots, for instance circular plots. The use of the package is covered in it's vignette.

15.3 *shiny* for easy interactive reports

As a final example of visualization, the *shiny* package and web site [1] has recently been introduced. It offers a new model for developing interactive, browser-based visualizations. These visualizations could be an excellent way to provide sophisticated exploratory or summary analysis in a very accessible way. The idea is to write a 'user interface' component that describes how a page is to be presented to users, and a 'server' that describes how the data are to be calculated or modified in responses to user choices. The programming model is 'reactive', where changes in a user choice automatically trigger re-calculations in the server. This reactive model is like in a spreadsheet with a formula, where adjusting a cell that the formula references

[1] http://www.rstudio.com/shiny/

Figure 15.1: *Gviz* ideogram, genome coordinate, annotation, and data tracks.



triggers re-calculation of the formula. Just like in a spreadsheet, someone creating a *shiny* application does not have to work hard to make reactivity work.

WARNING: The following demos were only available during the course.

There are two short demos available for use during the course. The first

```
> source("http://bioconductor.org/scratch-repos/pkgInstall.R")
> demo1()
```

Uses a *SummarizedExperiment* object to contain the data and results of the *DESeq* work flow from yesterday, coupled with the annotation resources that we explored today. The user can choose genes to display based on p-value and log-fold change in the 'top table' of genes. In reaction to these choices, the heat map on one tab and annotations on a second, are updated.

One of the nice features of *shiny* is it separates responsibility for doing manipulations of the data (*R*'s responsibility) from obligations to display it (*javascript*'s responsibility). This means that it can be quite 'easy' to incorporate complicate visualizations, as illustrated in

```
> demo2()
```

This represents a gene network under various conditions. The data is in *R*, and changing the slider causes *R* to update the data, but the visualization uses the javascript development version of *cytoscape*. This offers great opportunities for interaction with different projects.

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Part V

Appendix

Appendix A

DESeq vignette

Appendix B

VariantTools vignette