Aligning Short Sequencing Reads with Bowtie

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ABSTRACT

This unit shows how to use the Bowtie package to align short sequencing reads, such as those output by second-generation sequencing instruments. It also includes protocols for building a genome index and calling consensus sequences from Bowtie alignments using SAMtools. *Curr. Protoc. Bioinform.* 32:11.7.1-11.7.14. © 2010 by John Wiley & Sons, Inc.

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INTRODUCTION

The Bowtie (Langmead et al., 2009) package enables ultrafast and memory-efficient alignment of large sets of sequencing reads to a reference sequence, such as the human genome. The package contains tools for building indexes of reference genomes and for aligning short reads using the index as a guide. This is the first step of many comparative genomics workflows, including variant detection and digital gene expression. In what follows, the term *read* refers to a short DNA sequence, typically as output by a sequencing instrument. A read may be accompanied by a corresponding string of *quality values*, where each value estimates the probability that the corresponding base was miscalled by the instrument software. The term *subject sequence* refers to the true sequence of the sample(s) from which the reads were drawn. The term *reference sequence* or *reference genome* refers to the sequence to which the subject is to be compared. An example of a commonly used reference sequence is the public human genome assembly GRCh37.

To achieve both speed and memory efficiency, Bowtie aligns reads with the aid of an *index* of the reference genome. The Bowtie index is a refinement of the FM Index (Ferragina and Manzini, 2000), which uses the Burrows-Wheeler Transform (Burrows and Wheeler, 1994) to achieve both speed and space efficiency. The user must have built, or otherwise obtained, an appropriate index before reads can be aligned to the reference. Once an index is built, it can be queried any number of times. Indexes for commonly used reference genomes are also available for download from the Bowtie Web site at *http://bowtie-bio.sf.net*.

The Basic Protocol describes how to use the Bowtie tool to align a collection of reads to a reference genome, assuming an index is available. The indexing protocol (Alternate Protocol 1) describes how to build an index for a user-specified reference genome with the `bowtie-build` tool. The consensus and SNP calling protocol (Alternate Protocol 2) describes how to call consensus sequences, including SNPs, from Bowtie's output using SAMtools (Li et al., 2009). The command-line option protocol (Alternate Protocol 3) demonstrates a variety of commonly used alignment options. Finally, the Support Protocols describe how to obtain and install Bowtie software (Support Protocol 1), how to build Bowtie from source code (Support Protocol 2), and how to obtain pre-built indexes from the Bowtie Web site (Support Protocol 3). Each of these protocols

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runs in a variety of Unix or Unix-like environments, including Linux, Mac OS X, and Windows.

Bowtie is open source and can be used free of charge, subject to the terms of the Artistic License at *http://www.opensource.org/licenses/artistic-license-1.0.php*.

BASIC PROTOCOL

ALIGNING A SET OF SHORT READS TO A REFERENCE GENOME

This protocol uses the Bowtie tool to take a collection of short reads and search for each read's best alignment to a reference genome. In typical comparative genomics applications, an alignment is interpreted as a hypothesis for where the read originated in the genome. Once reads are aligned to the reference, the resulting set of alignments becomes a source of evidence for other features of interest, such as DNA sequence differences or gene expression measurements.

Bowtie has many command-line options that allow the user to tune its behavior according to the inputs and the desired result. This protocol describes a few commonly used options and gives some advice regarding how other options should be set. For a complete description of Bowtie's command-line options, see the `MANUAL` file included in the Bowtie package.

The first example uses an *E. coli* index that comes with the Bowtie package. Alternate Protocol 1 describes how to build an index from a user-specified reference genome.

All Bowtie options are specified on the command line when invoking Bowtie. A full list of Bowtie options can be found in the `MANUAL` file included with the Bowtie package; these examples focus on the shorter list of important options shown in Table 11.7.1.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

Software

Bowtie 0.12.5 package (see Support Protocols 1 and 2 for download and installation instructions)

Files

A set of one or more files containing the reads. Reads can be in the FASTA format (see *APPENDIX 1B* for information on FASTA), FASTQ format (Cock et al., 2010), or in a raw one-sequence-per-line format. The reads used in this example are a set of simulated reads included in the Bowtie package.

A set of index files containing the index of the reference genome. The index file format is unique to Bowtie, and FASTA formats are converted to this format using the Bowtie build tool (discussed in Alternate Protocol 1). The index files used for this example encode the whole genome of *E. coli* strain 536. These files are included in the Bowtie package.

Running Bowtie interactively from the command line

1. Switch to the directory containing Bowtie. For instance, if Bowtie is installed in a directory named `/Users/joe/software/bowtie`, type:

```
cd /Users/joe/software/bowtie
```

The example index files are located in the `indexes` *subdirectory, and reads are located in the* `reads` *subdirectory.*

Table 11.7.1 Descriptions of Important Command-Line Options for the Bowtie Alignment Tool[a]

Option	Description
Alignment policy	
`-v <int>`	Only alignments with up to `<int>` mismatches in the entire alignment are considered "valid." `-v` is mutually exclusive with `-n`. Default: `-n` mode is enabled and `-v` mode is disabled.
OR:	
`-n <int>`	Only alignments with up to `<int>` mismatches in the seed portion of the alignment are considered "valid." `-n` is mutually exclusive with `-v`. Default: 2.
`-l <int>`	When `-n` is specified, `-l` sets the length of the seed portion of the alignment to `<int>`. Default: 28.
`-e <int>`	When `-n` is specified, `-e` sets the limit on the sum of quality scores at the mismatched positions. Default: 70.
Reporting policy	
`-k <int>`	If Bowtie finds multiple valid alignments for a read, only report the first `<int>` alignments found. Default: 1.
`-m <int>`	If Bowtie finds more than `<int>` valid alignments for a read, suppress all alignments for the read. Default: no limit.
`-M <int>`	If Bowtie finds more than `<int>` valid alignments for a read, suppress the alignments but report one of them at random. Default: no limit.
Other options	
`-S`	Output alignments in SAM format (Li et al., 2009).
`-u <int>`	Stop and exit after the first `<int>` reads have been processed.

[a] For the full list of options, see the `MANUAL` file included with the version of Bowtie being used.

2. Run Bowtie on the example *E. coli* data with the `-u 10` command-line option:

```
./bowtie -u 10 indexes/e_coli reads/e_coli_1000.fq
```

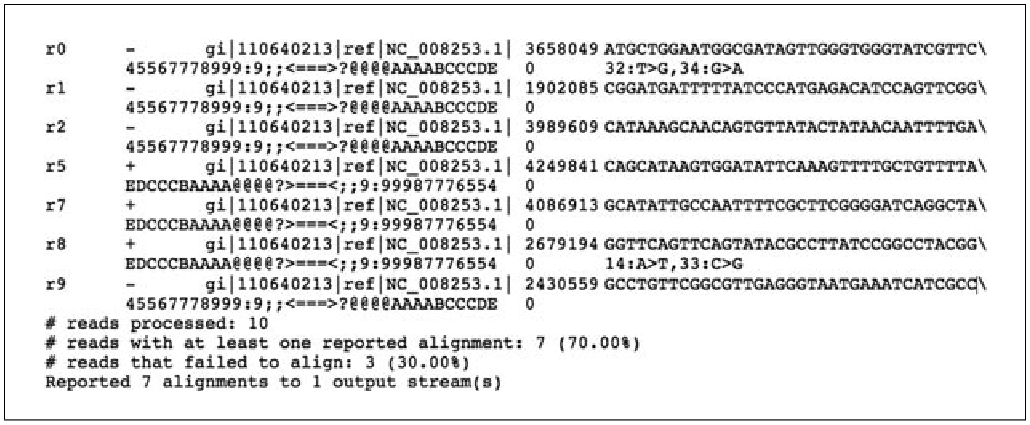
When invoking Bowtie, the user specifies an index file `basename`, *i.e., the prefix shared by all the index files (*`indexes/e_coli` *in this case), and the path(s) to the FASTQ read files to be aligned (*`reads/e_coli_1000.fq` *in this case). All other arguments to Bowtie are interpreted as options (see Table 11.7.1 for a list of a few important options).*

This command aligns all reads in the specified FASTQ file to the E. coli 536 reference genome, using the specified index as a guide. The `-u 10` *option causes Bowtie to exit after processing the first ten reads from the file.*

3. Examine and interpret the output, also shown in Figure 11.7.1.

Note that in practice, users rarely need to examine the alignment output manually. Rather, users will typically redirect Bowtie's output to a file or directly to another program for downstream processing. See Alternate Protocol 2 for an example.

The output consists of two sets of lines, those printed to the `standard out` *file handle and those printed to the* `standard error` *file handle. Each line printed to* `standard out` *is a valid, reportable alignment found by Bowtie. A valid alignment is one that satisfies the alignment policy specified in the* `-n`, `-l`, `-e`, *or* `-v` *options. A reportable alignment is one that is not suppressed by any other option, such as the* `-k`, `-m`, *or* `-M` *options. Lines printed to* `standard error` *contain summary information about the entire alignment run, e.g., the total number of reads that were processed and the percentage of total reads for which Bowtie found at least one alignment.*



```
r0      -       gi|110640213|ref|NC_008253.1|  3658049 ATGCTGGAATGGCGATAGTTGGGTGGGTATCGTTC\
        45567778999:9;;<===>?@@@@AAAABCCCDE    0       32:T>G,34:G>A
r1      -       gi|110640213|ref|NC_008253.1|  1902085 CGGATGATTTTTATCCCATGAGACATCCAGTTCGG\
        45567778999:9;;<===>?@@@@AAAABCCCDE    0
r2      -       gi|110640213|ref|NC_008253.1|  3989609 CATAAAGCAACAGTGTTATACTATAACAATTTTGA\
        45567778999:9;;<===>?@@@@AAAABCCCDE    0
r5      +       gi|110640213|ref|NC_008253.1|  4249841 CAGCATAAGTGGATATTCAAAGTTTTGCTGTTTTA\
        EDCCCBAAAA@@@@?>===<;;9:99987776554    0
r7      +       gi|110640213|ref|NC_008253.1|  4086913 GCATATTGCCAATTTTCGCTTCGGGGATCAGGCTA\
        EDCCCBAAAA@@@@?>===<;;9:99987776554    0
r8      +       gi|110640213|ref|NC_008253.1|  2679194 GGTTCAGTTCAGTATACGCCTTATCCGGCCTACGG\
        EDCCCBAAAA@@@@?>===<;;9:99987776554    0       14:A>T,33:C>G
r9      -       gi|110640213|ref|NC_008253.1|  2430559 GCCTGTTCGGCGTTGAGGGTAATGAAATCATCGCC\
        45567778999:9;;<===>?@@@@AAAABCCCDE    0
# reads processed: 10
# reads with at least one reported alignment: 7 (70.00%)
# reads that failed to align: 3 (30.00%)
Reported 7 alignments to 1 output stream(s)
```

Figure 11.7.1 Sample Bowtie session. The last few lines are written to the `standard error` file handle and they convey summary information about the run. The other lines are written to the `standard out` file handle and show valid, reportable alignments found by Bowtie. In this and subsequent figures, the backslash character is used to indicate that some long lines are wrapped.

The format for an alignment consists of eight fields, separated by tabs. The fields, from left to right, are:

a. Read name (`r0` in the case of the first alignment shown in Fig. 11.7.1).

b. Reference strand aligned to: "+" denotes forward strand, "−" denotes reverse strand ("−" for the first alignment in Fig. 11.7.1).

c. Name of the reference sequence aligned to (`gi|110640213|ref|NC_008253.1|` for the first alignment in Fig. 11.7.1).

d. Offset of the leftmost position on the forward reference strand covered by the alignment (`3658049` for the first alignment in Fig. 11.7.1).

e. Read sequence aligned, or its reverse complement if the read aligned to the reverse strand (`ATGCTG...` for the first alignment in Fig. 11.7.1).

f. Quality sequence aligned, or its reverse if the read aligned to the reverse strand (`455677...` for the first alignment in Fig. 11.7.1).

g. See the `MANUAL` file included with the Bowtie package for information on the seventh field (`0` for the first alignment in Fig. 11.7.1).

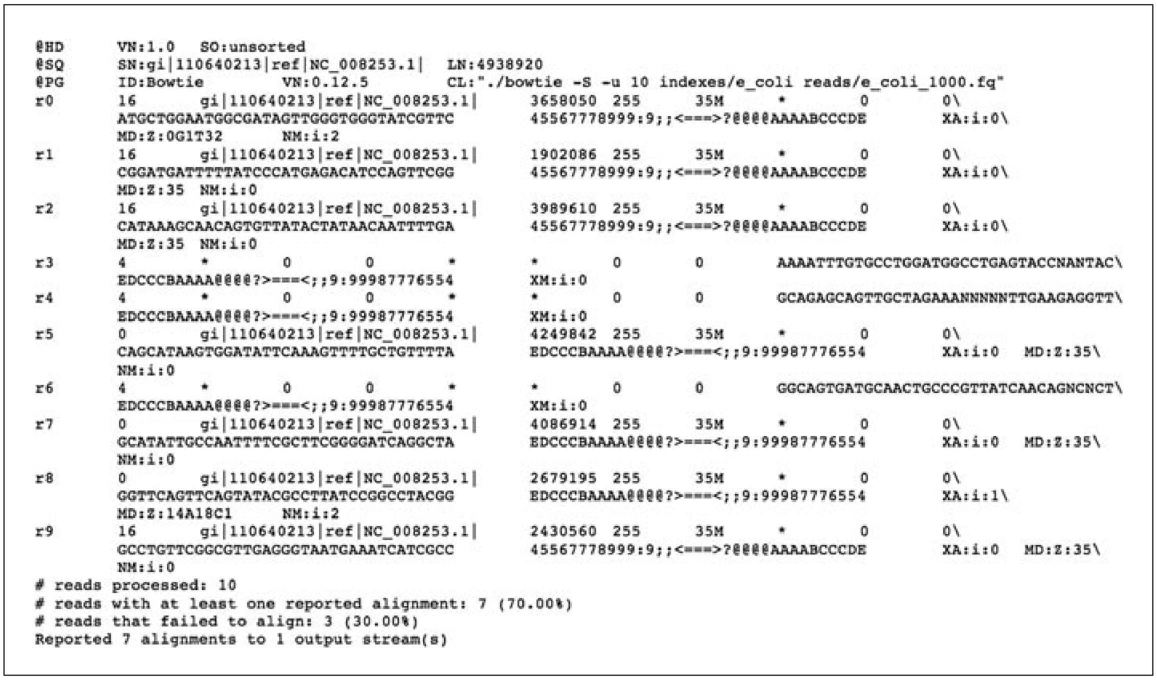
h. A string representing the differences between the read and the corresponding characters of the reference genome (`32:T>G,34:G>A` for the first alignment in Fig. 11.7.1). Each difference is described in a separate, comma-separated field.

4. To obtain alignment results in SAM format, a file format recognized by SAMTools and many other sequence analysis packages, add the `-S` option to the bowtie command-line:

```
./bowtie -S -u 10 indexes/e_coli reads/e_coli_1000.fq
```

This will produce output similar to the earlier example, except that the alignments printed to standard output will be in SAM format (Fig. 11.7.2). More information about the SAM format is available in the `MANUAL` *file included with the Bowtie package and on the SAMtools Web site at http://samtools.sourceforge.net/.*

5. When aligning a large number of reads, the user will wish to capture the standard output to a file rather than to have it appear on the screen. The user can do this by redirecting standard output as shown here:



```
@HD      VN:1.0   SO:unsorted
@SQ      SN:gi|110640213|ref|NC_008253.1|   LN:4938920
@PG      ID:Bowtie        VN:0.12.5       CL:"./bowtie -S -u 10 indexes/e_coli reads/e_coli_1000.fq"
r0       16       gi|110640213|ref|NC_008253.1|     3658050  255      35M      *        0        0\
         ATGCTGGAATGGCGATAGTTGGGTGGGTATCGTTC       45567778999:9;;<===>?@@@@AAAABCCCDE        XA:i:0\
         MD:Z:0G1T32      NM:i:2
r1       16       gi|110640213|ref|NC_008253.1|     1902086  255      35M      *        0        0\
         CGGATGATTTTTATCCCATGAGACATCCAGTTCGG       45567778999:9;;<===>?@@@@AAAABCCCDE        XA:i:0\
         MD:Z:35  NM:i:0
r2       16       gi|110640213|ref|NC_008253.1|     3989610  255      35M      *        0        0\
         CATAAAGCAACAGTGTTATACTATAACAATTTTGA       45567778999:9;;<===>?@@@@AAAABCCCDE        XA:i:0\
         MD:Z:35  NM:i:0
r3       4        *        0        0        *        *        0        0        AAAATTTGTGCCTGGATGGCCTGAGTACCNANTAC\
         EDCCCBAAAA@@@@?>===<;;9:99987776554       XM:i:0
r4       4        *        0        0        *        *        0        0        GCAGAGCAGTTGCTAGAAANNNNNTTGAAGAGGTT\
         EDCCCBAAAA@@@@?>===<;;9:99987776554       XM:i:0
r5       0        gi|110640213|ref|NC_008253.1|     4249842  255      35M      *        0        0\
         CAGCATAAGTGGATATTCAAAGTTTTGCTGTTTTA       EDCCCBAAAA@@@@?>===<;;9:99987776554        XA:i:0   MD:Z:35\
         NM:i:0
r6       4        *        0        0        *        *        0        0        GGCAGTGATGCAACTGCCCGTTATCAACAGNCNCT\
         EDCCCBAAAA@@@@?>===<;;9:99987776554       XM:i:0
r7       0        gi|110640213|ref|NC_008253.1|     4086914  255      35M      *        0        0\
         GCATATTGCCAATTTTCGCTTCGGGGATCAGGCTA       EDCCCBAAAA@@@@?>===<;;9:99987776554        XA:i:0   MD:Z:35\
         NM:i:0
r8       0        gi|110640213|ref|NC_008253.1|     2679195  255      35M      *        0        0\
         GGTTCAGTTCAGTATACGCCTTATCCGGCCTACGG       EDCCCBAAAA@@@@?>===<;;9:99987776554        XA:i:1\
         MD:Z:14A18C1     NM:i:2
r9       16       gi|110640213|ref|NC_008253.1|     2430560  255      35M      *        0        0\
         GCCTGTTCGGCGTTGAGGGTAATGAAATCATCGCC       45567778999:9;;<===>?@@@@AAAABCCCDE        XA:i:0   MD:Z:35\
         NM:i:0
# reads processed: 10
# reads with at least one reported alignment: 7 (70.00%)
# reads that failed to align: 3 (30.00%)
Reported 7 alignments to 1 output stream(s)
```

Figure 11.7.2 Sample Bowtie session when SAM output mode is enabled. The last few lines are written to the `standard error` file handle and convey summary information about the run and are not part of the SAM format. The first few lines beginning with `@` are the SAM header. The remaining lines are alignments.

```
./bowtie -S indexes/e_coli reads/e_coli_1000.fq >
  alignments.sam
```

This will align all reads contained in the E. coli FASTQ file and save them in SAM format to the file named `alignments.sam`.

BUILDING AN INDEX FOR A SET OF REFERENCE SEQUENCES

ALTERNATE PROTOCOL 1

This protocol uses the `bowtie-build` tool to take a collection of FASTA files for a reference genome and generate a collection of index files. Index files can then be used by Bowtie to align reads to the reference genome. The same set of index files can be used across multiple runs of Bowtie.

`bowtie-build` has command-line options that allow the user to tune its behavior, but this protocol will use the defaults. For a complete description of the `bowtie-build`'s command-line options, see the `MANUAL` file included with the Bowtie package.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

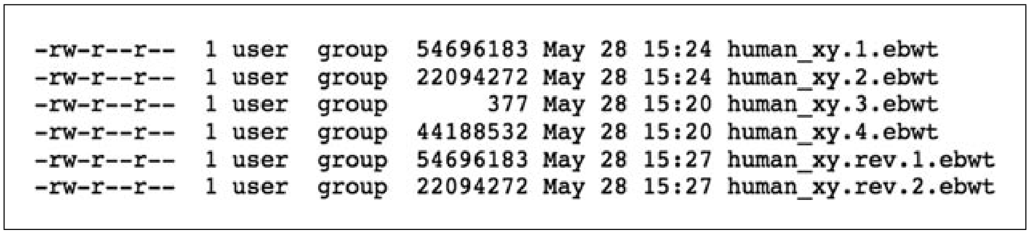
Software

Bowtie 0.12.5 package (see Support Protocols 1 and 2 for download and installation instructions)

1. Download the two compressed FASTA files corresponding to the human sex chromosomes (`chrX.fa.gz` and `chrY.fa.gz`) from the UCSC FTP site at *http://hgdownload.cse.ucsc.edu/goldenPath/hg19/chromosomes/*.

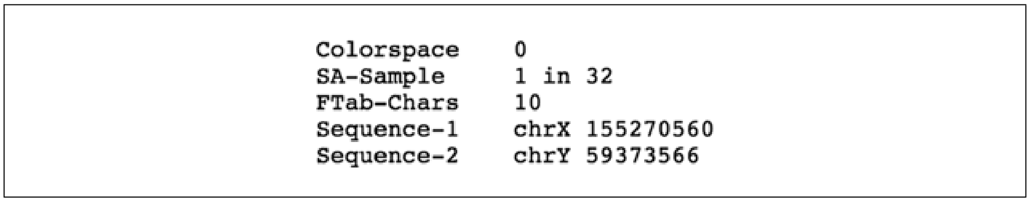
2. In the same directory, use gunzip to decompress the compressed FASTA files that were just downloaded:

```
gunzip *.gz
```



```
-rw-r--r--  1 user  group  54696183 May 28 15:24 human_xy.1.ebwt
-rw-r--r--  1 user  group  22094272 May 28 15:24 human_xy.2.ebwt
-rw-r--r--  1 user  group       377 May 28 15:20 human_xy.3.ebwt
-rw-r--r--  1 user  group  44188532 May 28 15:20 human_xy.4.ebwt
-rw-r--r--  1 user  group  54696183 May 28 15:27 human_xy.rev.1.ebwt
-rw-r--r--  1 user  group  22094272 May 28 15:27 human_xy.rev.2.ebwt
```

Figure 11.7.3 Listing of files output by `bowtie-build` after indexing the human sex chromosomes.



```
Colorspace    0
SA-Sample     1 in 32
FTab-Chars    10
Sequence-1    chrX 155270560
Sequence-2    chrY 59373566
```

Figure 11.7.4 Output of `bowtie-inspect` when inspecting the index consisting of the two human sex chromosomes.

3. Run `bowtie-build` to build an index of the two human sex chromosomes:

```
bowtie-build chrX.fa,chrY.fa human_xy
```

This builds an index consisting of the sequences in the `chrX.fa` *and* `chrY.fa` *files and stores the results in a set of six files with prefix* `human_xy`*. If the* `bowtie-build` *executable is not in the search path, specify the full path to* `bowtie-build` *instead. This command typically takes ~10 to 15 min.*

In this example, each FASTA file contains one sequence. FASTA files with multiple sequences ("multi-FASTA" files) are also permitted, in which case all sequences in the multi-FASTA file are included as separate sequences in the index.

4. Confirm that the output files were created:

```
ls -l human_xy*
```

The output should be similar to Figure 11.7.3. Note the index consists of six distinct files with the same prefix (`human_xy`*) and suffix (*`.ebwt`*).*

5. Inspect the index using `bowtie-inspect`:

```
bowtie-inspect -s human_xy
```

The output should be similar to Figure 11.7.4. Among other things, the output tells the user that the index is not for colorspace alignment (first line), and shows the names and lengths of the two reference sequences included in the index (last two lines). See the `MANUAL` *file included with the Bowtie package for detailed description of* `bowtie-inspect`*'s output.*

ALTERNATE PROTOCOL 2

ALIGNMENT AND VARIATION DETECTION USING Bowtie AND SAMtools

A common task in comparative genomics is to take a collection of sequencing reads derived from genomic DNA and obtain a list of variants, or differences between the subject's genome sequence and the reference genome sequence. This protocol outlines how to accomplish this using the *E. coli* index and simulated *E. coli* reads that come with the Bowtie package, together with the SAMtools package.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

Software

Bowtie 0.12.5 package (see Support Protocol 1 for download and installation instructions)

SAMtools 0.1.7 package (see instructions on the SAMtools Web site at *http://samtools.sourceforge.net* for how to download and install SAMtools)

Running Bowtie

1. Switch to the directory containing Bowtie. For instance, if Bowtie is installed in a directory named `/Users/joe/software/bowtie`, type:

```
cd /Users/joe/software/bowtie
```

2. Align the reads to the *E. coli* genome with Bowtie and save the SAM output to a temporary file:

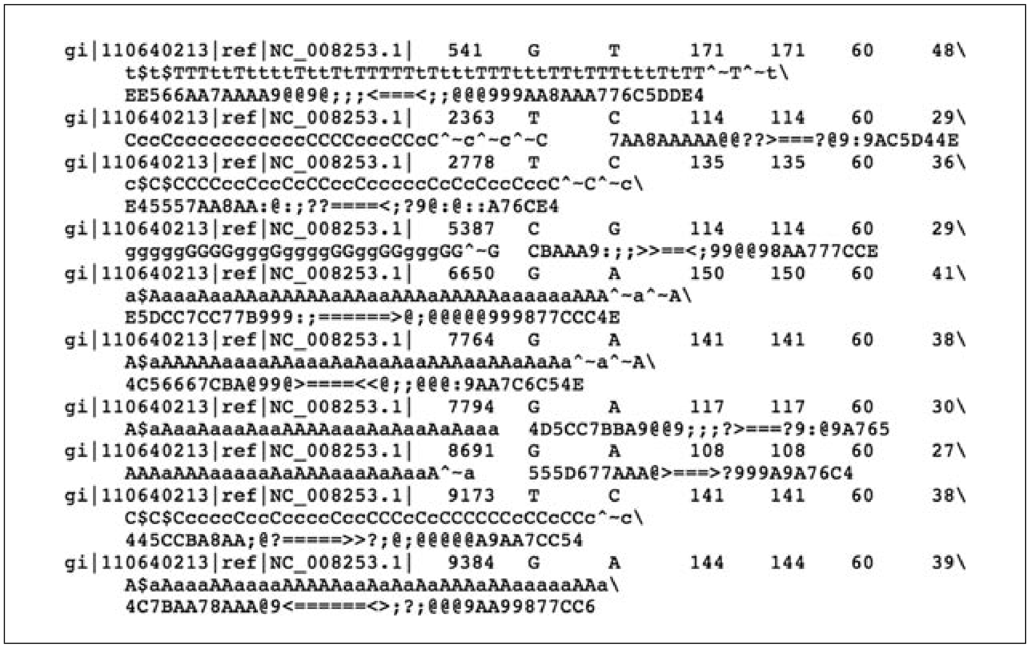
```
./bowtie -S indexes/e_coli reads/e_coli_10000snp.fq >
  /tmp/e_coli.sam
```

3. Convert the SAM output to BAM format:

```
samtools view -bS -o /tmp/e_coli.bam /tmp/e_coli.sam
```

BAM is the compressed, binary encoded counterpart to SAM.

If the `samtools` *executable is not in the search path, specify the full path to* `samtools` *instead.*



```
gi|110640213|ref|NC_008253.1|   541    G      T      171    171    60     48\
     t$t$TTTTttTttttTttTtTTTTTtTtttTTTtttTTtTTTttttTtTT^-T^-t\
     EE566AA7AAAA9@@9@;;;<===<;;@@@999AA8AAA776C5DDE4
gi|110640213|ref|NC_008253.1|   2363   T      C      114    114    60     29\
     CccCcccccccccccCCCCccccCCcC^-c^-c^-C     7AA8AAAAA@@??>===?@9:9AC5D44E
gi|110640213|ref|NC_008253.1|   2778   T      C      135    135    60     36\
     c$C$CCCCcccCccCcCCccCccccccCcCcCccCcccC^-C^-c\
     E45557AA8AA:@:;??====<;?9@:@::A76CE4
gi|110640213|ref|NC_008253.1|   5387   C      G      114    114    60     29\
     gggggGGGGgggGggggGGggGGgggGG^-G  CBAAA9:;;>>==<;99@@98AA777CCE
gi|110640213|ref|NC_008253.1|   6650   G      A      150    150    60     41\
     a$AaaaAaaAAaAAAAAaAAaaAAAaAAAAAaaaaaaaAAA^-a^-A\
     E5DCC7CC77B999:;======>@;@@@@@999877CCC4E
gi|110640213|ref|NC_008253.1|   7764   G      A      141    141    60     38\
     A$aAAAAAaaaaAAaaaAaAaaAaaAAAaaAAaAaAa^-a^-A\
     4C56667CBA@99@>====<<@;;@@@:9AA7C6C54E
gi|110640213|ref|NC_008253.1|   7794   G      A      117    117    60     30\
     A$aAaaAaaaAaaAAAAaaaAaAaaAaAaaa  4D5CC7BBA9@@9;;;?>===?9:@9A765
gi|110640213|ref|NC_008253.1|   8691   G      A      108    108    60     27\
     AAAaAAAaaaaaAaAAAaaaAaAaaA^-a    555D677AAA@>===>?999A9A76C4
gi|110640213|ref|NC_008253.1|   9173   T      C      141    141    60     38\
     C$C$CccccCccCccccCccCCCcCcCCCCCCcCCcCCc^-c\
     445CCBA8AA;@?=====>>?;@;@@@@@A9AA7CC54
gi|110640213|ref|NC_008253.1|   9384   G      A      144    144    60     39\
     A$aAaaaAAaaaaaAAAAAaaAaAaAaAAAaAAaaaaaAAa\
     4C7BAA78AAA@9<======<>;?;@@@9AA99877CC6
```

Figure 11.7.5 Output of the SAMtools consensus caller when calling SNPs from a simulated *E. coli* example dataset.

4. Convert the BAM file to a sorted BAM file:

```
samtools sort /tmp/e_coli.bam /tmp/e_coli.sorted
```

Sorted BAM is a useful format because alignments are both compressed, which is convenient for long-term storage, and sorted, which is convenient for variant discovery and other downstream analyses.

If the `samtools` *executable is not in the search path, specify the full path to* `samtools` *instead.*

5. Call variants from the sorted BAM file:

```
samtools pileup -cvf genomes/NC_008253.fna
  /tmp/e_coli.sorted.bam
```

The output should be similar to Figure 11.7.5, consisting of ten SNPs. See the SAMtools Web site at http://samtools.sourceforge.net for details about SAMtools output and command-line options.

ALTERNATE PROTOCOL 3

RUNNING Bowtie WITH VARIOUS COMMAND-LINE OPTIONS

Table 11.7.1 shows a short list of important command-line options that modify the behavior of the Bowtie alignment tool. This protocol steps through a series of examples that illustrate some of these options. The protocol uses the *E. coli* index that comes with the Bowtie package.

Besides the options being demonstrated, these examples use Bowtie's `--suppress` option, which suppresses unneeded output fields. In addition, the summary output that is normally printed to the `standard error` file is omitted from the figures in this protocol. The purpose is to keep the figures as concise as possible.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

Software

Bowtie 0.12.5 package (see Support Protocols 1 and 2 for download and installation instructions)

1. Switch to the directory containing Bowtie. For instance, if Bowtie is installed in a directory named `/Users/joe/software/bowtie`, type:

```
cd /Users/joe/software/bowtie
```

2. Align a test string to the *E. coli* genome using the `-a` and `-v 2` options:

```
./bowtie -a -v 2 e_coli --suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.6. The `--suppress` *option has suppressed all output fields besides the strand (first column), offset (second column), and edit string (third column).*

Specifying `-a`*, instructs Bowtie to report all valid alignments subject to the alignment policy, which is* `-v 2` *in this case. Here,* `Bowtie` *finds five inexact hits in the E. coli genome; one hit (the second listed) has one mismatch, and the other four hits have two mismatches. Four are on the reverse reference strand and one is on the forward strand. Note that they are not listed in best-to-worst order.*

3. Align a test string to the *E. coli* genome using the `-k 3` and `-v 2` options:

```
./bowtie -k 3 -v 2 e_coli --suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.7. Specifying `-k 3` *instructs Bowtie to report up to three valid alignments. In this case, a total of five valid alignments exist (see Fig. 11.7.6); Bowtie reports three out of those five.* `-k` *can be set to any integer greater than 0.*

4. Align a test string to the *E. coli* genome using the `-k 6` and `-v 2` options:

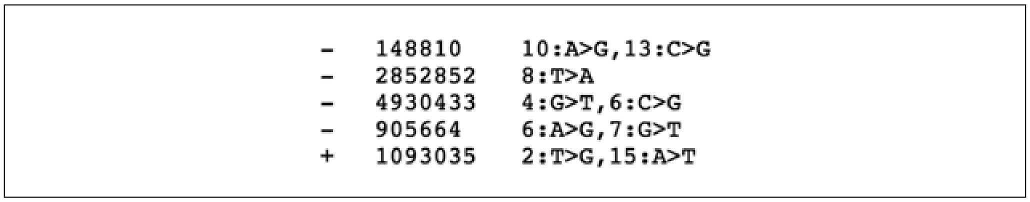
```
./bowtie -k 6 -v 2 e_coli --suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.8. Specifying `-k 6` *instructs Bowtie to report up to six valid alignments. In this case, a total of five valid alignments exist (see Fig. 11.7.6), so Bowtie reports all five.*

5. Align a test string to the *E. coli* genome using just the `-v 2` option:

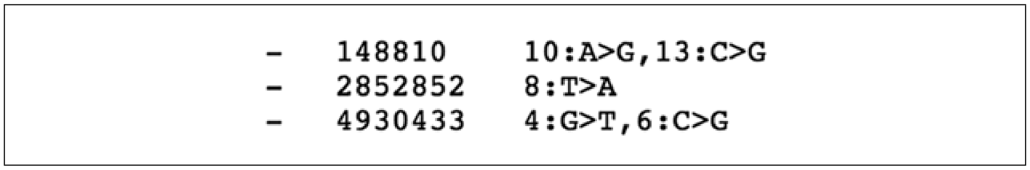
```
./bowtie -v 2 e_coli --suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.9. Leaving the reporting options at their defaults causes Bowtie to report the first valid alignment it encounters. Because `--best` *was not specified, we are not guaranteed that Bowtie will report the best alignment, and in this case, it does not (the 1-mismatch alignment from Fig. 11.7.8 would have been better). The default reporting mode is equivalent to* `-k 1`*.*



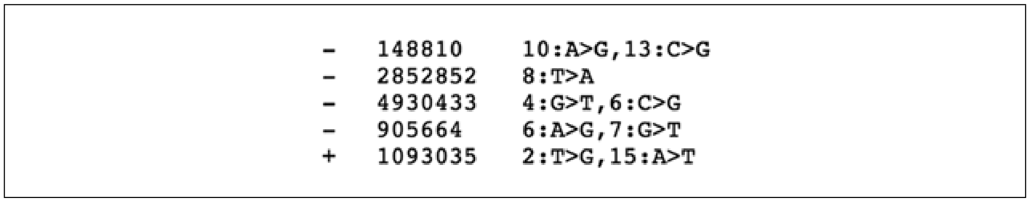
```
-   148810    10:A>G,13:C>G
-   2852852   8:T>A
-   4930433   4:G>T,6:C>G
-   905664    6:A>G,7:G>T
+   1093035   2:T>G,15:A>T
```

Figure 11.7.6 Bowtie output for the previous example using `-a` and `-v 2` options.



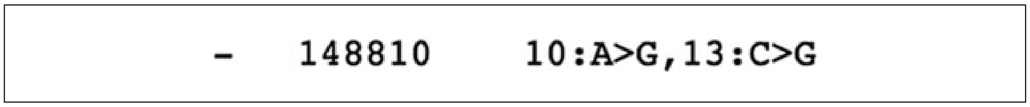
```
-   148810    10:A>G,13:C>G
-   2852852   8:T>A
-   4930433   4:G>T,6:C>G
```

Figure 11.7.7 Bowtie output for the previous example using `-k 3` and `-v 2` options.



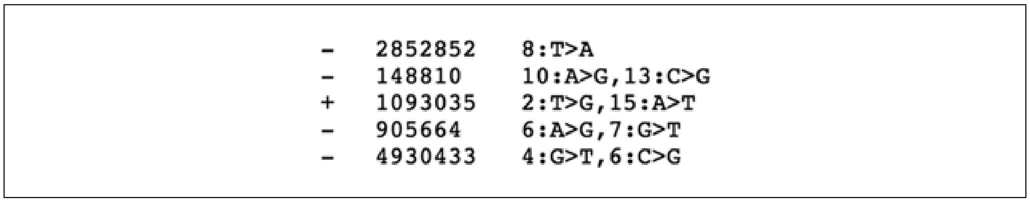
```
-   148810    10:A>G,13:C>G
-   2852852   8:T>A
-   4930433   4:G>T,6:C>G
-   905664    6:A>G,7:G>T
+   1093035   2:T>G,15:A>T
```

Figure 11.7.8 Bowtie output for the previous example using `-k 6` and `-v 2` options.



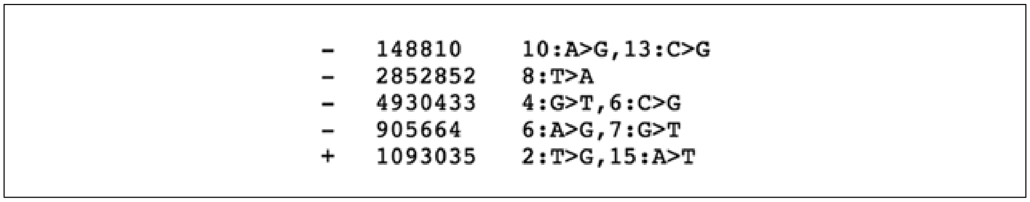
```
-   148810    10:A>G,13:C>G
```

Figure 11.7.9 Bowtie output for the previous example using just the `-v 2` option.



```
-   2852852   8:T>A
-   148810    10:A>G,13:C>G
+   1093035   2:T>G,15:A>T
-   905664    6:A>G,7:G>T
-   4930433   4:G>T,6:C>G
```

Figure 11.7.10 Bowtie output for the previous example using the `--best`, `-a`, and `-v 2` options.



```
-   148810    10:A>G,13:C>G
-   2852852   8:T>A
-   4930433   4:G>T,6:C>G
-   905664    6:A>G,7:G>T
+   1093035   2:T>G,15:A>T
```

Figure 11.7.11 Bowtie output for the previous example using the `-a` and `-m 5` and `-v 2` options.

6. Align a test string to the *E. coli* genome using the `--best`, `-a` and `-v 2` options:

```
./bowtie -a --best -v 2 e_coli --suppress 1,3,5,6,7
  -c ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.10. The `-a` *option causes all valid alignments to be printed and the* `--best` *option guarantees that Bowtie will find and print the alignments for a given read in best-to-worst order. In this case, the 1-mismatch alignment is printed first, as expected.*

7. Align a test string to the *E. coli* genome using the `-a`, `-m 3`, and `-v 2` options:

```
./bowtie -a -m 3 -v 2 e_coli —suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

There should be no alignment output. The `-a` *option causes Bowtie to report all valid alignments, but the* `-m 3` *option suppresses all alignments for reads with more than three valid alignments. Because this read has five valid alignments (see Fig. 11.7.10), all alignments are suppressed and there is no alignment output.*

8. Align a test string to the *E. coli* genome using the `-a` and `-m 5` and `-v 2` options:

```
./bowtie -a -m 5 -v 2 e_coli —suppress 1,3,5,6,7 -c
  ATGCATCATGCGCCAT
```

The output should be similar to Figure 11.7.11. The `-a` *option causes Bowtie to report all valid alignments while the* `-m 5` *option suppresses all alignments for reads with more than five valid alignments. Because this read has exactly five valid alignments, all alignments are reported.*

SUPPORT PROTOCOL 1

OBTAINING AND INSTALLING THE Bowtie PACKAGE

The Bowtie binary packages are available from the Bowtie Web site at *http://bowtie-bio.sf.net*. See the Latest Release section on the right-hand side of the Bowtie home page for a link to the appropriate Sourceforge download page. All Bowtie packages are compressed in the zip format.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation.

Software

Bowtie 0.12.5 package (see Support Protocol 1 for download and installation instructions)
A tool for extracting zip files

1. Download the desired Bowtie binary package from the Bowtie Web site at *http://bowtie-bio.sf.net*.
2. Unzip the downloaded file to the desired installation path.
3. *Optional:* For convenience and compatibility with other tools that rely on Bowtie, add the extracted directory (containing the `bowtie` and `bowtie-build` executables) to the search path.

   Users unfamiliar with how to add directories to the search path should consult APPENDIX 1C*.*

SUPPORT PROTOCOL 2

BUILDING Bowtie FROM SOURCE

The Bowtie source packages are available from the Bowtie Web site at *http://bowtie-bio.sf.net*. See the Latest Release section on the right-hand side of the Bowtie home page for a link to the appropriate Sourceforge download page. The source package filenames end with `-src.zip`. All Bowtie packages are compressed in the zip format.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

Software

Bowtie 0.12.5 package (see Support Protocol 1 for download and installation instructions)
A tool for extracting zip files
Standard GNU development tools, including g++ and GNU make

1. Download the desired Bowtie binary package from the Bowtie Web site at *http://bowtie-bio.sf.net*.
2. Unzip the downloaded file to the desired installation path.
3. Switch to the extracted directory and run the make command:

```
cd (extracted directory)
make
```

   The build process requires that standard development tools, including `g++`*, GNU make, and* `find` *be installed, and libraries, such as* `libpthread` *may also need to be installed. See the included* `MANUAL` *file for more details about how to build Bowtie.*

   When the build process completes successfully, a set of binaries including `bowtie`*,* `bowtie-build`*, and* `bowtie-inspect` *will be created in the extracted directory.*

4. *Optional:* For convenience and compatibility with other tools that rely on Bowtie, add the extracted directory (containing the `bowtie` and `bowtie-build` executables) to the search path.

   Users unfamiliar with how to add directories the search path should consult APPENDIX 1C*.*

SUPPORT PROTOCOL 3

DOWNLOADING AND INSTALLING A PREBUILT Bowtie INDEX

The Bowtie source packages are available from the Bowtie Web site at *http://bowtie-bio.sf.net*. See the "Pre-built indexes" section on the right-hand side of the Bowtie home page for download links. Indexes are compressed in the zip format.

This protocol steps through obtaining and using a prebuilt index for the *S. cerevisiae* genome.

Necessary Resources

Hardware

A Unix, Linux, Mac OS X, or Windows 32-bit workstation

Software

Bowtie 0.12.5 package (see Support Protocol 1 for download and installation instructions)
A tool for extracting zip files

1. Download the prebuilt index for the *S. cerevisiae* genome by visiting the Bowtie Web site at *http://bowtie-bio.sf.net* and clicking the link labeled "*S. cerevisiae*" on the right-hand side of the home page. Place the downloaded file in a temporary directory.
2. Unzip the downloaded file to the desired path.
3. Run a simple Bowtie query against the new index as a test:

```
bowtie (index install path)/s_cerevisiae -c
  ATATATATATATATA
```

If the `bowtie` *executable is not in the search path, specify the full path to* `bowtie` *instead. In this case,* `bowtie` *should print one alignment.*

GUIDELINES FOR UNDERSTANDING RESULTS

The purpose of alignment is to determine the reads' *point of origin* with respect to the reference genome. Once points of origin are identified, downstream tools use that information, e.g., to characterize differences between the subject and reference genome (e.g., when calling SNPs), or to relate the reads to annotations defined with respect to the reference genome (e.g., for digital gene expression). Alignment programs, together with appropriate reference sequences, serve this purpose because genomes of individuals of the same species tend to be highly similar. For example, two humans typically have ~3 to 4 million single-nucleotide differences between them out of a total of 3 billion bases. However, comparative strategies also have inherent drawbacks that should be kept in mind when interpreting Bowtie results.

Repetitive genome content affects results

Some genomes, including the human genome, have substantial repetitive content, i.e., sub-sequences that appear multiple times throughout the genome. Repeats come in several forms (e.g., simple repeats, tandem repeats, segmental duplications, and interspersed repeats), and arise via various biological processes (e.g., slipped strand mispairing or retrotransposition). Repeats also affect alignments because reads originating from repetitive portions of the genome are difficult or impossible to *unambiguously* assign to a point of origin. Reads from repeats will tend to have many "valid" alignments, with no strong basis for preferring one over the others. Paired-end reads mitigate but do not necessarily eliminate this problem. Repetitive alignments, in turn, affect downstream analyses. For instance, if ambiguous alignments are included in the output from Bowtie, a SNP could yield false positives and false negatives purely owing to the repeat structure.

The simplest way to deal with alignment ambiguity is to use Bowtie's `-m` and `-M` options to filter out and/or annotate ambiguous evidence as such. When ambiguous alignments are annotated in this way, downstream tools can choose to discount or ignore evidence from ambiguous alignments as appropriate. With the `-m` option, the user specifies a limit whereby alignments for reads that align to a number of locations exceeding the limit are excluded from the output. For instance, to suppress all alignments for reads that align to more than three locations on the reference, the user specifies `-m 3`. The `-M` option is similar to the `-m` option except that one random alignment from among those found is reported, but is marked as being ambiguous. See the `MANUAL` file included in the Bowtie package for details.

Sequence differences between subject and reference affect results

Though human genomes are close to 99.9% similar at the sequence level, the differences can have a significant effect on alignment. For instance, if the subject genome contains a cluster of three single-nucleotide variants within ten bases of each other, and Bowtie is run with the `-v 2` option (i.e., only alignments with up to two mismatches are valid), Bowtie is very unlikely to report the correct alignment for a read whose true point of origin spans the three variants. A similar example involves gaps, if the subject genome has a gap with respect to the reference genome (or vice versa), Bowtie is unlikely to report the correct alignment for a read whose true point of origin spans the gap. These potential effects must be taken into account when interpreting output from Bowtie.

COMMENTARY

Background Information

The Bowtie package enables ultrafast and memory-efficient alignment of large sets of sequencing reads to a reference sequence. Alignment poses a serious computational challenge because the increase in throughput provided by second-generation sequencing instruments is rapidly outpacing the growth in computer speeds. Alignment is also important for biological research because it typically constitutes both the first and the slowest step of comparative genomics pipelines.

Bowtie aligns reads with the aid of an index of the reference genome, and the indexing technique used in Bowtie is the key to its speed and memory efficiency. The Bowtie index is a refinement of the FM Index (Ferragina and Manzini, 2000), which in turn uses the Burrows-Wheeler Transform (Burrows and Wheeler, 1994). The user must have built, or otherwise obtained, an appropriate index before reads can be aligned to the reference, but once an index is built, it can be queried any number of times. Alternatively, the user can download a prebuilt index from the Bowtie Web site at *http://bowtie-bio.sf.net*.

Critical Parameters and Troubleshooting

The most often-used Bowtie options are discussed in the protocols above, but `bowtie` and `bowtie-build` both support dozens of options that help the user achieve good results for a breadth of inputs. This section describes some additional families of options that are useful under certain circumstances; see the `MANUAL` file included in the Bowtie package for a complete listing of options.

If either sensitivity (i.e., percent of reads that align) and/or speed are an issue, try adjusting the alignment policy. Alignment policy is controlled either by the `-v` option (in end-to-end mode) or by the `-n`, `-l`, and `-e` options (in seeded mode). For greater speed but lower sensitivity, try the following adjustments as appropriate: adjust `-v` down, `-n` down, `-l` up, and `-e` down. For lower speed but greater sensitivity, make the opposite adjustments.

For greater speed without sacrificing sensitivity, try the `-p` option. Specifying a value for `-p` >1 is a convenient way to allow Bowtie to use more than one computer processor. When multiple processors are available and Bowtie is run with `-p` equal to the number of available processors, Bowtie will take proportionally less time to complete.

If you receive an error message about memory having been exhausted, try adjusting the `--offrate` option up, first to 6, then higher. If that does not work, try running on a computer with more memory.

The `-C` option, when passed to `bowtie`, enables alignment of colorspace reads, such as those produced by the ABI SOLiD instrument. When specified to `bowtie-build`, the `-C` option causes `bowtie-build` to generate a *colorspace index*, rather than a normal index (also called a *base space* or *nucleotide space* index). When the `-C` option is passed to `bowtie`, the specified index must be a colorspace index. Likewise, if the `-C` option is not passed to `bowtie`, the specified index must be a normal index.

For alignment of paired-end or mate-paired reads, use the `-1` and `-2` options to specify the paired input files. See the `--ff`, `--fr`, `--rf` options for information on read orientations. Note that the default for non-colorspace reads is `--fr`, since this matches the output of the Illumina instruments' most commonly used paired-end protocol. Likewise, `--ff` is the default for colorspace reads, since this matches the output of the ABI SOLiD instrument's most commonly used protocol. Also, see the `-I` and `-X` options, which place bounds on the permitted fragment size.

Finally, the `--al`, `--un`, and `--max` options can be a convenient way to prepare for the use of a downstream tool. Each option takes a filename argument. If all three are specified, Bowtie, in addition to printing alignment output as usual, will print each read to one of the three specified files according to the alignment result. If one or more alignments were reported for the read, the read is printed to the file specified with the `--al` option. If no alignments were found for the read, the read is printed to the file specified with the `--un` option. If alignments were found, but were suppressed due to the `-m` or `-M` options, the read is printed to the file specified with the `--max` option. These files can then be used as input to another analysis; e.g., the reads that failed to align could then be passed to a spliced aligner or to another invocation of Bowtie using a different index.

Literature Cited

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Cock, P.J., Fields, C.J., Goto, N., Heuer, M.L., and Rice, P.M. 2010. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. *Nucleic Acids Res.* 38:1767-1771.

Ferragina, P. and Manzini, G. 2000. Opportunistic data structures with applications. *In* Proceedings of the 41st Annual Symposium on Foundations of Computer Science. IEEE Computer Society.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-2079.

Key Reference

Langmead et al., 2009. See above.

Describes the indexing technique underlying the tool and compares to other alignment tools.

Internet Resource

http://bowtie-bio.sf.net

The project's home page where the latest version of the package can be downloaded and where announcements are made.