Working with FASTQ files

Setup

Logon and idev

First login to **ls6** like you did before. Then start an **idev** session so that we don't do too much processing on the login nodes.

Start an idev session

```
idev -m 120 -N 1 -A OTH21164 -r CoreNGS-Tue
# -or-
idev -m 90 -N 1 -A OTH21164 -p development
```

Data staging

Set ourselves up to process some yeast data data in **$SCRATCH**, using some of best practices for organizing our workflow.

Set up directory for working with FASTQs

```
# Create a $SCRATCH area to work on data for this course,
# with a sub-directory for pre-processing raw fastq files
mkdir -p $SCRATCH/core_ngs/fastq_prep

# Make symbolic links to the original yeast data:
cd $SCRATCH/core_ngs/fastq_prep
ln -s -f $CORENGS/yeast_stuff/Sample_Yeast_L005_R1.cat.fastq.gz
ln -s -f $CORENGS/yeast_stuff/Sample_Yeast_L005_R2.cat.fastq.gz

# or
ln -s -f ~/CoreNGS/yeast_stuff/Sample_Yeast_L005_R1.cat.fastq.gz
ln -s -f ~/CoreNGS/yeast_stuff/Sample_Yeast_L005_R2.cat.fastq.gz

# or
ln -sf /work/projects/BioITeam/projects/courses/Core_NGS_Tools/yeast_stuff/Sample_Yeast_L005_R1.cat.fastq.gz
ln -sf /work/projects/BioITeam/projects/courses/Core_NGS_Tools/yeast_stuff/Sample_Yeast_L005_R2.cat.fastq.gz
```

Illumina sequence data format (FASTQ)

GSAF gives you paired end sequencing data in two matching **FASTQ** format files, containing reads for each end sequenced. See where your data really is and how big it is.

ls options to see the size of linked files

```
# the -l options says "long listing" which shows where the link goes,
# but doesn't show details of the real file
ls -l

# the -L option says to follow the link to the real file,
#     -l means long listing (includes size)
#     -h says "human readable" (e.g. MB, GB)
ls -Llh
```

4-line FASTQ format

Each read end sequenced is represented by a 4-line entry in the **FASTQ** file that looks like this:

A four-line FASTQ file entry representing one sequence

```
@HWI-ST1097:127:C0W5VACXX:5:1101:4820:2124 1:N:0:CTCAGA
TCTCTAGTTTCGATAGATTGCTGATTTGTTTCCTGGTCATTAGTCTCCGTATTTATATTATTATCCTGAGCATCATTGATGGCTGCAGGAGGAGCATTCTC
+
CCCFFFFDHHHHHGGGHIJIJJIHHJJJHHIGHHIJFHGICA91CGIGB?9EF9DDBFCGIGGIIIID>DCHGCEDH@C@DC?3AB?@B;AB??;=A>3;;
```

Line 1 is the ***unique read name*****.** The format for Illumina reads is as follows, using the read name above:

machine_id:lane:flowcell_grid_coordinates end_number:failed_qc:0:barcode

@HWI-ST1097:127:C0W5VACXX:5:1101:4820:2124 1:N:0:CTCAGA

- The line ***as a whole*** will be unique for this read fragment.
  - the corresponding R1 and R2 reads will have identical **machine_id:lane:flowcell_grid_coordinates** information. This common part of the name ties the two read ends together
  - the **end_number:failed_qc:0:barcode** information will be different for R1 and R2
- Most sequencing facilities will not give you qc-failed reads (**failed_qc = Y**) unless you ask for them.

Line 2 is the ***sequence*** reported by the machine, starting with the first base of the insert (the **5'** adapter has usually been removed by the sequencing facility). These are **ACGT** or **N** uppercase characters.

Line 3 always starts with '**+**' (it can optionally include a sequence description)

Line 4 is a string of ***Ascii-encoded base quality scores***, one character per base in the sequence.

- For each base, an integer ***Phred***-type quality score is calculated as **`integer score = -10 log(probabilty base is wrong)`** then added to 33 to make a number in the Ascii printable character range.
- As you can see from the table below, ***alphabetical letters - good***, ***numbers – ok***, **most special characters – bad** (except **:;<=>?@**).
- See **https://www.asciitable.com**

```
Quality character   !"#$%&'()*+,-./0123456789:;<=>?@ABCDEFGHIJ
                    |         |         |         |         |
ASCII Value         33        43        53        63        73
Base Quality (Q)    0         10        20        30        40
```

See the Wikipedia FASTQ format page for more information.

Exercise: What character in the quality score string in the FASTQ entry above represents the best base quality? Roughly what is the error probability estimated by the sequencer?

J is the best base quality score character (Q=41)

It represents a probability of error of < 1/10^4 or 1/10,000

About compressed files

Sequencing data files can be very large - from a few megabytes to gigabytes. And with NGS giving us longer reads and deeper sequencing at decreasing price points, it's not hard to run out of storage space. As a result, most sequencing facilities will give you ***compressed*** sequencing data files.

The most common compression program used for ***individual files*** is **gzip** whose compressed files have the **.gz** extension. The **tar** and **zip** programs are most commonly used for compressing ***directories*.**

Let's take a look at the size difference between uncompressed and compressed files. We use the **-l** option of ls to get a **l**ong listing that includes the file size, and **-h** to have that size displayed in "**h**uman readable" form rather than in raw byte sizes.

Compare compressed and uncompressed files

```
ls -lh $CORENGS/yeast_stuff/*L005*.fastq
ls -lh $CORENGS/yeast_stuff/*L005*.fastq.gz
```

Pathname wildcarding

The asterisk character (*****) is a ***pathname wildcard*** that matches 0 or more characters.

Read more about pathname wildcards: **Pathname wildcards**

Exercise: About how big are the compressed files? The uncompressed files? About what is the compression factor?

FASTQ's are ~ 149 MB
Compressed they are ~ 50 MB
This is about 3x compression

You may be tempted to want to un-compress your sequencing files in order to manipulate them more directly – but resist that temptation! Nearly all modern bioinformatics tools are able to work on **.gz** files, and there are tools and techniques for working with the contents of compressed files without ever un-compressing them.

gzip and gunzip

With no options, **gzip** compresses the file you give it in-place. Once all the content has been compressed, the original uncompressed file is removed, leaving only the compressed version (the original file name plus a **.gz** extension). The **gunzip** function works in a similar manner, except that its input is a compressed file with a **.gz** file and produces an uncompressed file without the **.gz** extension.

gzip, gunzip exercise

```
# if the $CORENGS environment variable is not defined
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools

# make sure you're in your $SCRATCH/core_ngs/fastq_prep directory
cd $SCRATCH/core_ngs/fastq_prep

# Copy over a small, uncompressed fastq file
cp $CORENGS/misc/small.fq .

# check the size, then compress it in-place
ls -lh small*
gzip small.fq

# check the compressed file size
ls -lh small*

# uncompress it again
gunzip small.fq.gz
ls -lh small*
```

Both **gzip** and **gunzip** are ***extremely I/O intensive*** when run on large files.

While TACC has tremendous compute resources and its specialized parallel file system is great, it has its limitations. It is not difficult to overwhelm the TACC file system if you **gzip** or **gunzip** more than a few files at a time – as few as 5-6!

The intensity of compression/decompression operations is another reason you should compress your sequencing files once (if they aren't already) then leave them that way.

head and tail, more or less

One of the challenges of dealing with large data files, whether compressed or not, is finding your way around the data – finding and looking at relevant pieces of it. Except for the smallest of files, you can't open them up in a text editor because those programs read the whole file into memory, so will choke on sequencing data files! Instead we use various techniques to look at pieces of the files at a time. (Read more about commands for **Displaying file contents**)

The first technique is the use of ***pagers*** – we've already seen this with the **more** command. Review its use now on our small uncompressed file:

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
cp $CORENGS/misc/small.fq .
```

Using the more pager

```
# Use spacebar to advance a page; Ctrl-c to exit
more small.fq
```

Another pager, with additional features, is **less**. The most useful feature of **less** is the ability to search – but it still doesn't load the whole file into memory, so searching a really big file can be slow.

Here's a summary of the most common **less** navigation commands, once the **less** pager is active. It has tons of other options (try **less --help**).

- **q** – quit
- **Ctrl-f** or **space** – page forward
- **Ctrl-b** – page backward
- **/<pattern>** – search for **<pattern>** in ***forward*** direction
  - **n** – next match
  - **N** – previous match
- **?<pattern>** – search for **<pattern>** in ***backward*** direction
  - **n** – previous match going back
  - **N** – next match going forward

If you start **less** with the **-N** option, it will display line numbers.

Exercise: What line of small.fq contains the read name with grid coordinates 2316:10009:100563?

```
less -N small.fq
/2316:10009:100563
```

line number 905, which looks like this:

```
905 @HWI-ST1097:127:C0W5VACXX:5:2316:10009:100563 1:N:0:CTCAGA
```

head

For a really quick peek at the first few lines of your data, there's nothing like the **head** command. By default **head** displays the first 10 lines of data from the file you give it or from its ***standard input***. With an argument **-NNN** (that is a dash followed by some number), it will show that many lines of data.

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
cp $CORENGS/misc/small.fq .
```

Using the head command

```
# shows 1st 10 lines
head small.fq

# shows 1st 100 lines -- might want to pipe this to more to see a bit at a time
head -100 small.fq | more
```

So what if you want to see line numbers on your **head** or **tail** output? Neither command seems to have an option to do this.

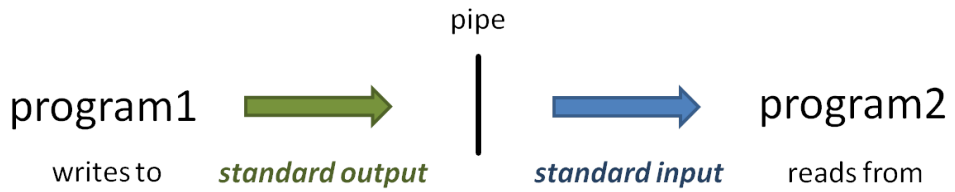
cat --help

```
cat -n small.fq | tail
```

piping

So what is that **vertical bar** (**|**) all about? It is the ***pipe operator***!

The ***pipe operator*** (**|**) connects one program's ***standard output*** to the next program's ***standard input***. The power of the Linux command line is due in no small part to the power of piping. (Read more about **Piping** and **Standard Unix I/O streams**)



- When you execute the **head -100** **small.fq** **| more** command, **head** starts writing lines of the **small.fq** file to ***standard output***.
- Because of the ***pipe***, the output does not go to the **Terminal**, but is connected to the ***standard input*** of the **more** command.
- Instead of reading lines from a file you specify as a command-line argument, **more** obtains its input from ***standard input***.
- The **more** command writes a page of text to ***standard output***, which is displayed on the **Terminal**.

Programs often allow input from standard input

Most Linux commands are designed to accept input from ***standard input*** in addition to (or instead of) command line arguments so that data can be ***piped in***.

Many bioinformatics programs also allow data to be piped in. Often they will require you provide a special argument, such as **stdin** or **-**, to tell the program data is coming from ***standard input*** instead of a file.

tail

The yang to **head**'s ying is **tail**, which by default it displays the ***last*** 10 lines of its data, and also uses the **-NNN** syntax to show the last **NNN** lines. (Note that with very large files it may take a while for **tail** to start producing output because it has to read through the file sequentially to get to the end.)

But what's really cool about **tail** is its **-n +NN** syntax. This displays all the lines starting at line **NN**. Note this syntax: the **-n** option switch follows by a plus sign (**+**) in front of a number – the plus sign is what says "starting at this line"! Try these examples:

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
cp $CORENGS/misc/small.fq .
```

Using the tail command

```
# shows the last 10 lines
tail small.fq

# shows the last 100 lines -- might want to pipe this to more to see a bit at a time
tail -100 small.fq | more

# shows all the lines starting at line 900 -- better pipe it to a pager!
# cat -n adds line numbers to its output so we can see where we are in the file
cat -n small.fq | tail -n +900 | more

# shows 15 lines starting at line 900 because we pipe to head -15
tail -n +900 small.fq | head -15
```

zcat and gunzip -c tricks

Ok, now you know how to navigate an un-compressed file using **head** and **tail**, **more** or **less**. But what if your **FASTQ** file has been compressed by **gzip**? You don't want to un-compress the file, remember?

So you use the **gunzip -c** trick. This un-compresses the file, but instead of writing the un-compressed data to another file (without the **.gz** extension) it write it to its ***standard output*** where it can be piped to programs like your friends **head** and **tail**, **more** or **less**.

Let's illustrate this using one of the compressed files in your **fastq_prep** sub-directory:

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
cp $CORENGS/misc/small.fq .
```

Uncompressing output on the fly with gunzip -c

```
# make sure you're in your $SCRATCH/core_ngs/fastq_prep directory
cd $SCRATCH/core_ngs/fastq_prep

gunzip -c Sample_Yeast_L005_R1.cat.fastq.gz | more
gunzip -c Sample_Yeast_L005_R1.cat.fastq.gz | head
gunzip -c Sample_Yeast_L005_R1.cat.fastq.gz | tail
gunzip -c Sample_Yeast_L005_R1.cat.fastq.gz | tail -n +901 | head -8

# Note that less will display .gz file contents automatically
less -N Sample_Yeast_L005_R1.cat.fastq.gz
```

Finally, another command that does the same thing as **gunzip -c** is **zcat** – which is like **cat** except that it works on **gzip**-compressed (**.gz**) files!

Counting lines with wc -l

```
zcat Sample_Yeast_L005_R1.cat.fastq.gz | more
zcat Sample_Yeast_L005_R1.cat.fastq.gz | less -N
zcat Sample_Yeast_L005_R1.cat.fastq.gz | head
zcat Sample_Yeast_L005_R1.cat.fastq.gz | tail
zcat Sample_Yeast_L005_R1.cat.fastq.gz | tail -n +901 | head -8
# include original line numbers
zcat Sample_Yeast_L005_R1.cat.fastq.gz | cat -n | tail -n +901 | head -8
```

There will be times when you forget to pipe your large **zcat** or **gunzip -c** output somewhere – even the experienced among us still make this mistake! This leads to pages and pages of data spewing across your **Terminal**.

If you're lucky you can kill the output with **Ctrl-c**. But if that doesn't work (and often it doesn't) just close your **Terminal** window. This terminates the process on the server (like hanging up the phone), then you just can log back in.

Counting your sequences

One of the first thing to check is that your **FASTQ** files are the same length, and that length is evenly divisible by 4. The **wc** command (**w**ord **c**ount) using the **-l** switch to tell it to count **l**ines, not words, is perfect for this. It's so handy that you'll end up using **wc -l** a ***lot*** to count things. It's especially powerful when used with filename wild carding.

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
cp $CORENGS/misc/small.fq .
```

Counting lines with wc -l

```
wc -l small.fq
head -100 small.fq > small2.fq
wc -l small*.fq
```

You can also pipe the output of **zcat** or **gunzip -c** to **wc -l** to count lines in your compressed FASTQ file.

Exercise: How many lines are in the Sample_Yeast_L005_R1.cat.fastq.gz file? How many sequences is this?

```
zcat Sample_Yeast_L005_R1.cat.fastq.gz | wc -l
```

The **wc -l** command says there are 2,368,720 lines. FASTQ files have 4 lines per sequence, so the file has 2,368,720/4 or 592,180 sequences.

How to do math on the command line

The **bash** shell has a really strange syntax for arithmetic: it uses a double-parenthesis operator after the **$** sign (which means evaluate this expression). Go figure.

Syntax for artithmetic on the command line

```
echo $((2368720 / 4))
```

Here's another trick: ***backticks evaluation***. When you enclose a command expression in ***backtick quotes*** (`) the enclosed expression is evaluated and its ***standard output*** substituted into the string. (Read more about **Quoting in the shell**)

Here's how you would combine this math expression with **zcat** line counting on your file using the magic of backtick evaluation. Notice that the **wc -l** expression is what is reading from ***standard input***.

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
ln -sf $CORENGS/yeast_stuff/Sample_Yeast_L005_R1.cat.fastq.gz
ln -sf $CORENGS/yeast_stuff/Sample_Yeast_L005_R2.cat.fastq.gz
```

Counting sequences in a FASTQ file

```
cd $SCRATCH/core_ngs/fastq_prep
zcat Sample_Yeast_L005_R1.cat.fastq.gz | echo "$((`wc -l` / 4))"
```

Whew!

bash arithmetic is integer valued only

Note that arithmetic in the **bash** shell is integer valued only, so don't use it for anything that requires decimal places!

A better way to do math

Well, doing math in **bash** is pretty awful – there has to be something better. There is! It's called **awk**, which is a powerful scripting language that is easily invoked from the command line.

In the code below we pipe the output from **wc -l** (number of lines in the **FASTQ** file) to **awk**, which executes its ***body*** (the statements between the curly braces (**{ }**) for each line of input. Here the input is just one line, with one field – the line count. The **awk** body just divides the 1st input field (**$1**) by 4 and writes the result to ***standard output***. (Read more about **awk** in **Advanced commands: awk**)

```
# Setup (if needed)
export CORENGS=/work/projects/BioITeam/projects/courses/Core_NGS_Tools
mkdir -p $SCRATCH/core_ngs/fastq_prep
cd $SCRATCH/core_ngs/fastq_prep
ln -sf $CORENGS/yeast_stuff/Sample_Yeast_L005_R1.cat.fastq.gz
ln -sf $CORENGS/yeast_stuff/Sample_Yeast_L005_R2.cat.fastq.gz
```

Counting FASTQ sequences with awk

```
cd $SCRATCH/core_ngs/fastq_prep
zcat Sample_Yeast_L005_R1.cat.fastq.gz | wc -l | awk '{print $1 / 4}'
```

Note that **$1** means something different in **awk** – the 1st **whitespace**-delimited input field – than it does in **bash**, where it represents the 1st argument to a script or function (technically, the **1** environment variable). This is an example of where a **metacharacter** - the **dollar sign** (**$**) here – has a different meaning for two different programs.

The **bash** shell treats **dollar sign** (**$**) as an ***evaluation operator***, so will normally attempt to evaluate the environment variable name following the **$** and substitute its value in the output (e.g. **echo $SCRATCH**). But we don't want that evaluation to be applied to the **{print $1 / 4}** script argument passed to **awk** ; instead we want **awk** to see the literal string **{print $1 / 4}** as its script. To achieve this result we surround the script argument with **single quotes** (**' '**), which tells the shell to treat everything enclosed by the quotes as ***literal text***, and not perform any ***metacharacter evaluation***.

(Read more about **Quoting in the shell**)

Processing multiple compressed files

You've probably figured out by now that you can't easily use filename wildcarding along with **zcat** and piping to process multiple files. For this, you need to code a **for** loop in **bash**. Fortunately, this is pretty easy. Try this:

For loop to count sequences in multiple FASTQs

```
cd $SCRATCH/core_ngs/fastq_prep
for fname in *.gz; do
  echo "Processing $fname"
  echo "..$fname has `zcat $fname | wc -l | awk '{print $1 / 4}'` sequences"
done
```

Each time through the **for** loop, the next item in the ***argument list*** (here the files matching the ***wildcard glob* *.gz**) is assigned to the **for** loop's ***formal argument*** (here the variable **fname**). The actual filename is then referenced as **$fname** inside the loop. (Read more about **Bash control flow**)