RNA sequence data analysis via Galaxy, Part I Uploading data and starting the workflow (Group Exercise)

The goal of this exercise is to use a Galaxy workflow to analyze RNA sequencing data. The datasets we will us are all feely available through the sequence repositories (SRA, ENA, DDBJ).

Galaxy is an open, web-based platform for data intensive biomedical research. EuPathDB is developing its own Galaxy instance that will become available for its users. Galaxy allows you to perform, reproduce, and share complete analyses.

Many resources are available to learn how to use Galaxy. The following link has information about additional resources to help you learn how to use Galaxy:

https://wiki.galaxyproject.org/Learn#Galaxy_101

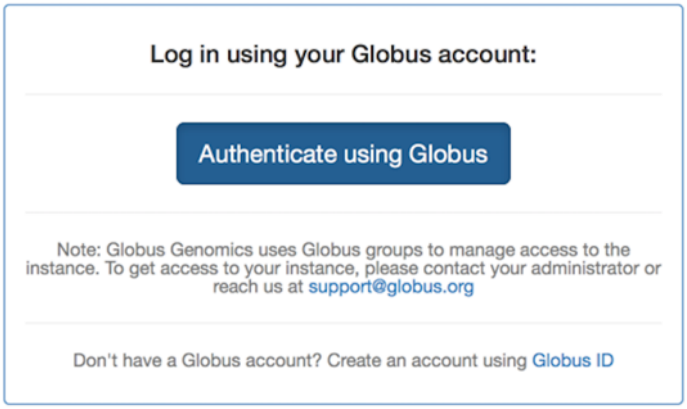
For this exercise we will be working in groups. Each group will have 4-6 members. One person in the group will run the Galaxy controls on one computer. The other members' role is to help ensure that the correct datasets are being used and that the correct workflow parameters are being selected.

The galaxy instance we will use is located here:

http://eupathdb.globusgenomics.org

Section I: Setting up your EuPathDB Galaxy account

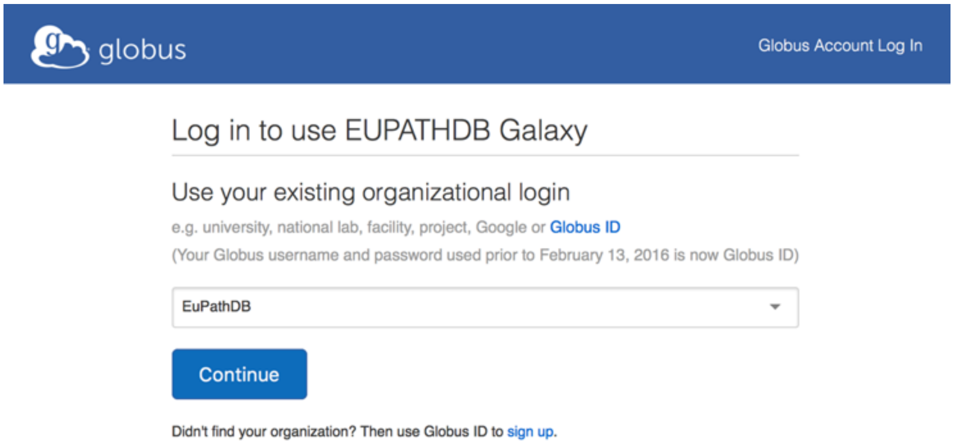
Step 1: Log in to any EuPathDB site.
Step 2: Go to the EuPathDB Galaxy instance: https://eupathdb.globusgenomics.org/
Step 3: Click on "Authenticate using Globus"


Log in using your Globus account:

Note: Globus Genomics uses Globus groups to manage access to the instance. To get access to your instance, please contact your administrator or reach us at support@globus.org

Don't have a Globus account? Create an account using Globus ID

Step 4: Select EuPathDB from the drop down menu if it is not already selected. Next click on "Continue"



Log in to use EUPATHDB Galaxy

Use your existing organizational login

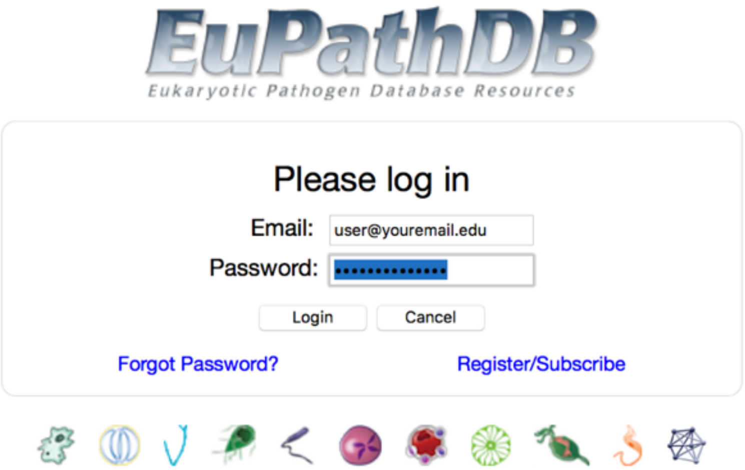
e.g. university, national lab, facility, project, Google or Globus ID

(Your Globus username and password used prior to February 13, 2016 is now Globus ID)

EuPathDB

Didn't find your organization? Then use Globus ID to sign up.

Step 5: Enter your EuPathDB user name and password.

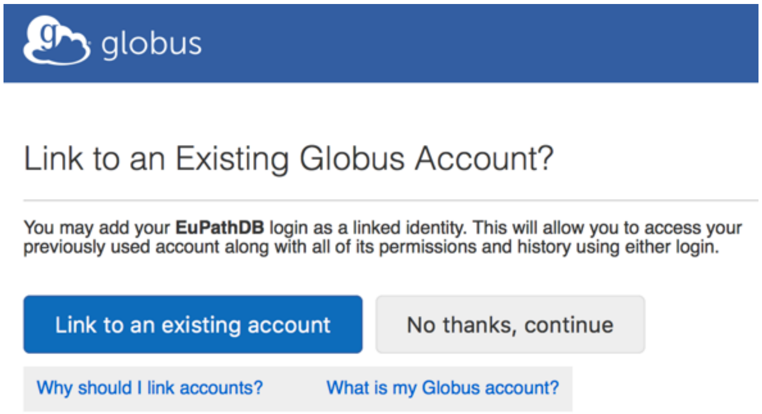


Please log in

Email: user@youremail.edu

Password: ••••••••••••••

Step 6: Click on "No thanks, continue"

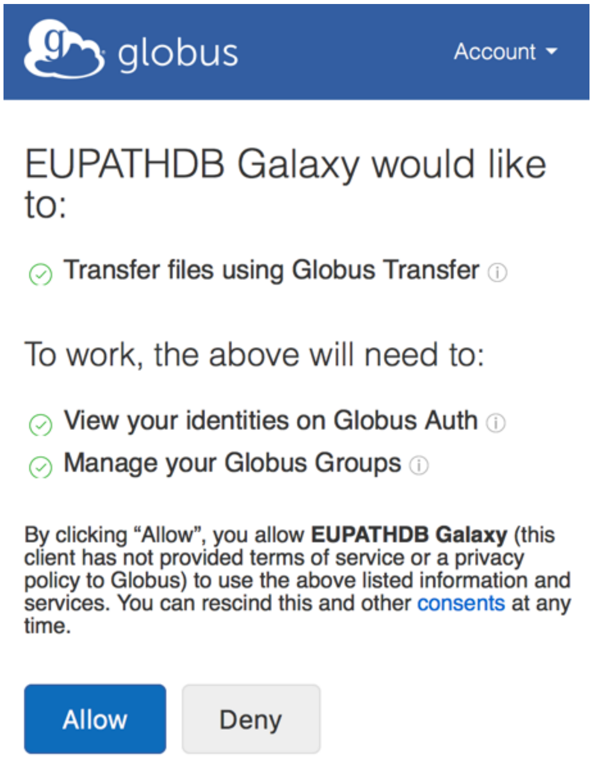


Link to an Existing Globus Account?

You may add your **EuPathDB** login as a linked identity. This will allow you to access your previously used account along with all of its permissions and history using either login.

Link to an existing account
No thanks, continue
Why should I link accounts?
What is my Globus account?

Step 7: Click on “Allow”



EUPATHDB Galaxy would like to:

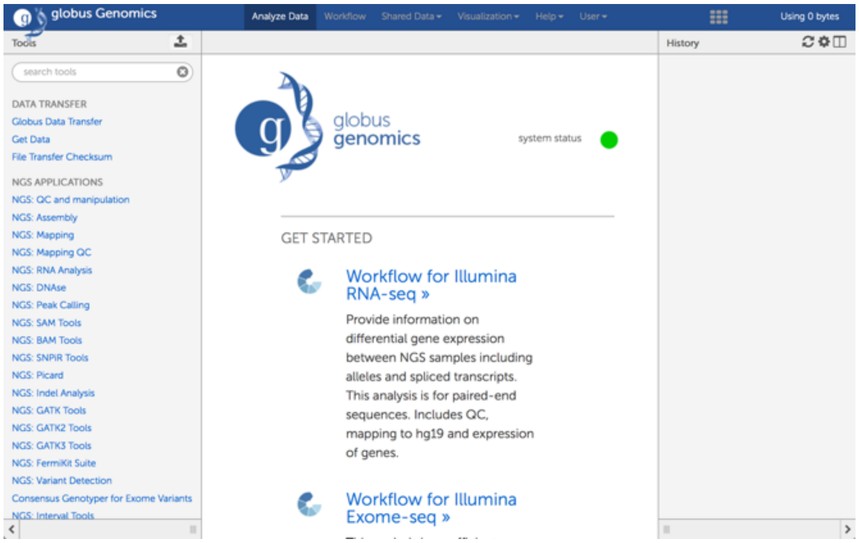
- Transfer files using Globus Transfer

To work, the above will need to:

- View your identities on Globus Auth
- Manage your Globus Groups

By clicking “Allow”, you allow **EUPATHDB Galaxy** (this client has not provided terms of service or a privacy policy to Globus) to use the above listed information and services. You can rescind this and other consents at any time.

Step 8: Congratulations, you are in!



GET STARTED

Workflow for Illumina RNA-seq »

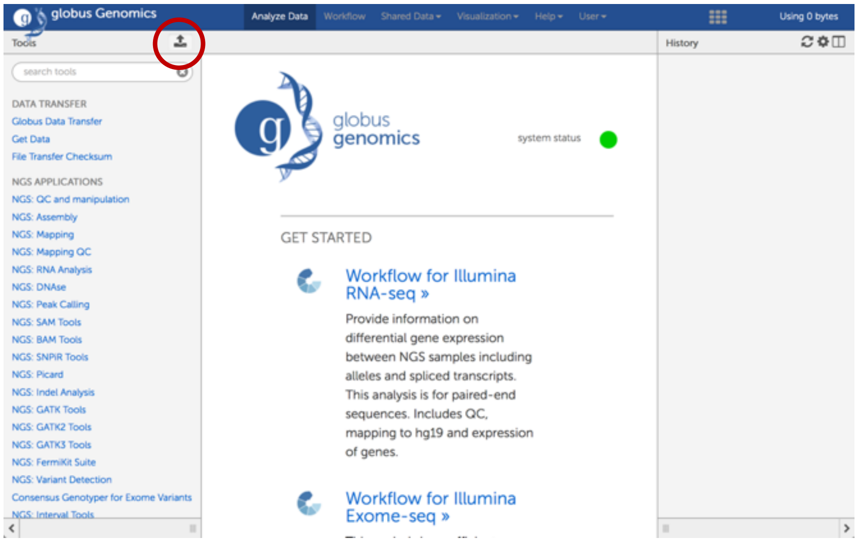
Provide information on differential gene expression between NGS samples including alleles and spliced transcripts. This analysis is for paired-end sequences. Includes QC, mapping to hg19 and expression of genes.

Workflow for Illumina Exome-seq »

Section II: Importing data to Galaxy

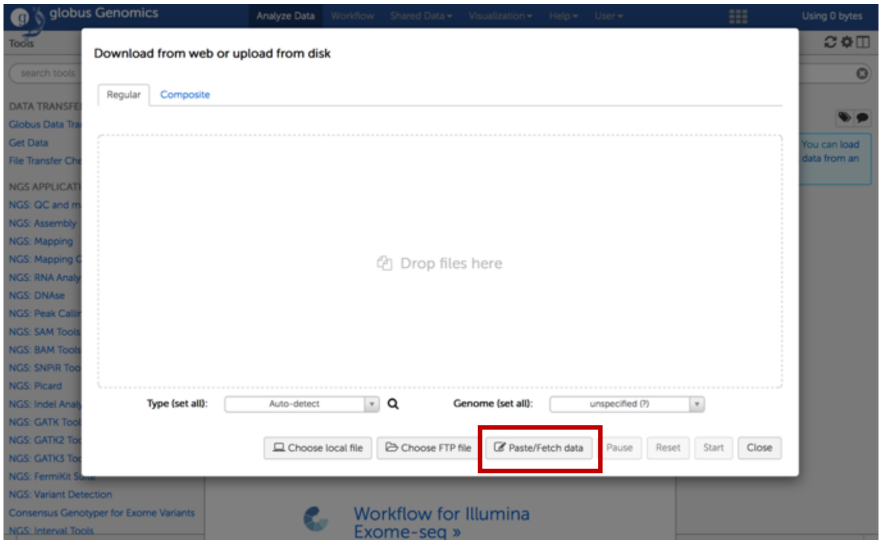
There are multiple ways to important data into your Galaxy workspace. For this exercise we will use the direct links listed below. Remember one person in your group will be doing this. The samples below were all generated by paired end sequencing, hence there are two files for each sample. The files are fastq files that are compressed (that is why they end in .gz = gzip).

Step 1: Click on the "Get data" icon. This will open up a window that allows you to "**Download from web or upload from disk"**

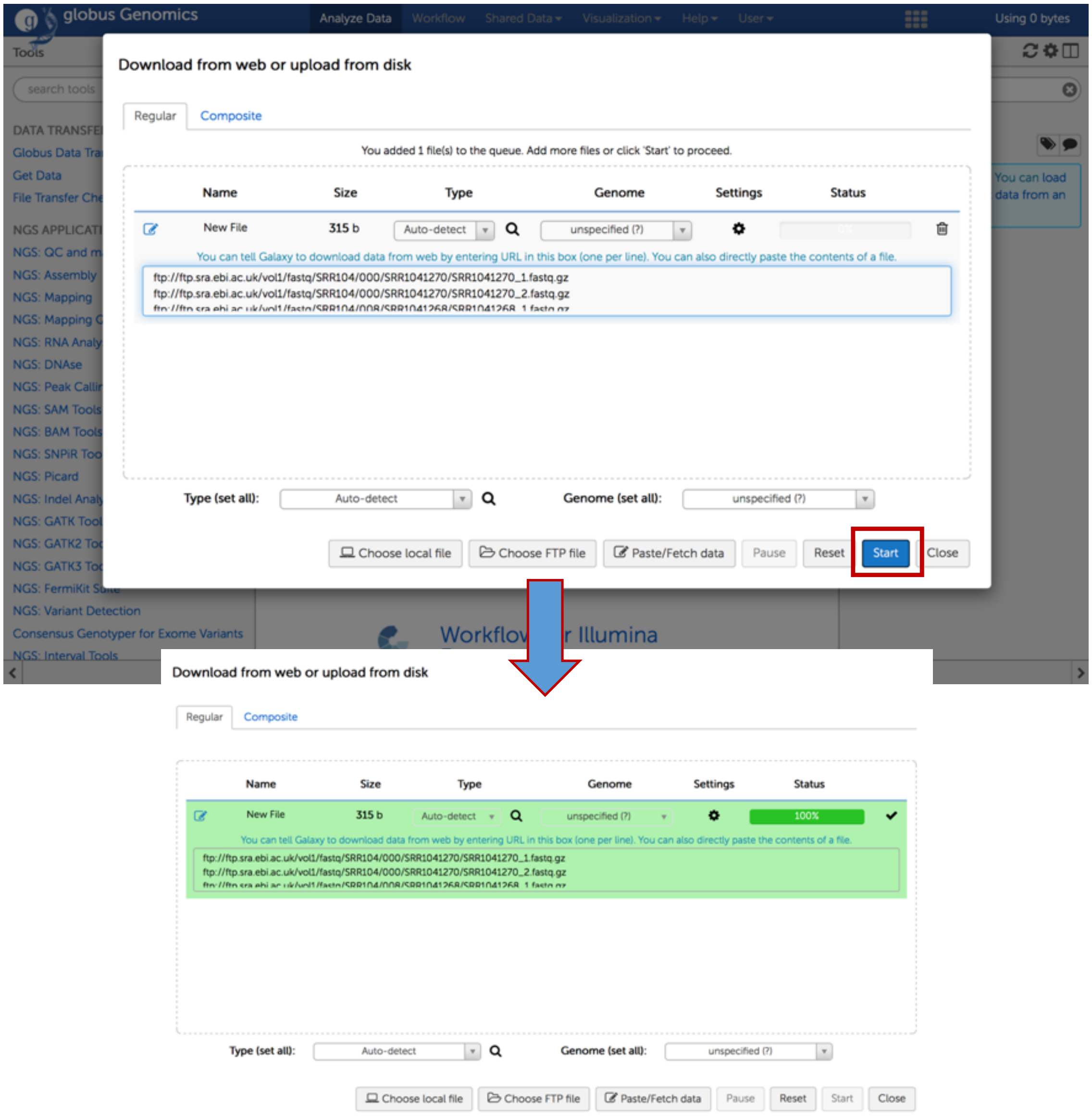


Provide information on differential gene expression between NGS samples including alleles and spliced transcripts. This analysis is for paired-end sequences. Includes QC, mapping to hg19 and expression of genes.

Step 2: In the "Download from web or upload from disk" window click on "Paste/Fetch data"



Step 3: Paste the four URLs corresponding to the four files for your group. Each URL has to be on a new line. Then click on “Start”.



Download from web or upload from disk

You added 1 file(s) to the queue. Add more files or click 'Start' to proceed.

Name	Size	Type	Genome	Settings	Status
New File	315 b	Auto-detect	unspecified (?)		

You can tell Galaxy to download data from web by entering URL in this box (one per line). You can also directly paste the contents of a file.

```
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz
```

Type (set all): Auto-detect

Genome (set all): unspecified (?)

Download from web or upload from disk

Name	Size	Type	Genome	Settings	Status
New File	315 b	Auto-detect	unspecified (?)		100% ✓

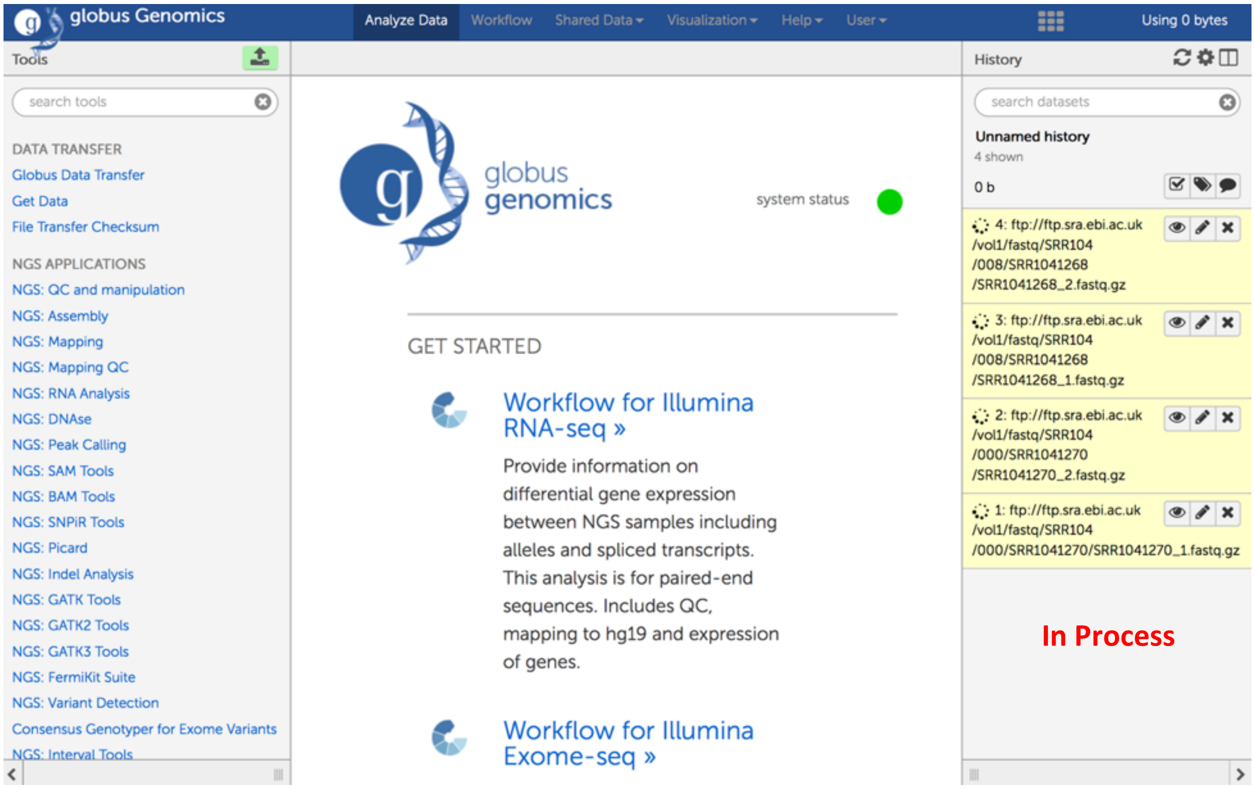
You can tell Galaxy to download data from web by entering URL in this box (one per line). You can also directly paste the contents of a file.

```
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz
```

Type (set all): Auto-detect

Genome (set all): unspecified (?)

Step 4: Click on “Close”. You should notice that the left section (history section) will show the files being transferred (yellow) – this may take a few minutes to start. File transfer will take about 15-20 minutes. When this is complete they will turn green.



Provide information on differential gene expression between NGS samples including alleles and spliced transcripts. This analysis is for paired-end sequences. Includes QC, mapping to hg19 and expression of genes.

Unnamed history
4 shown
0 b

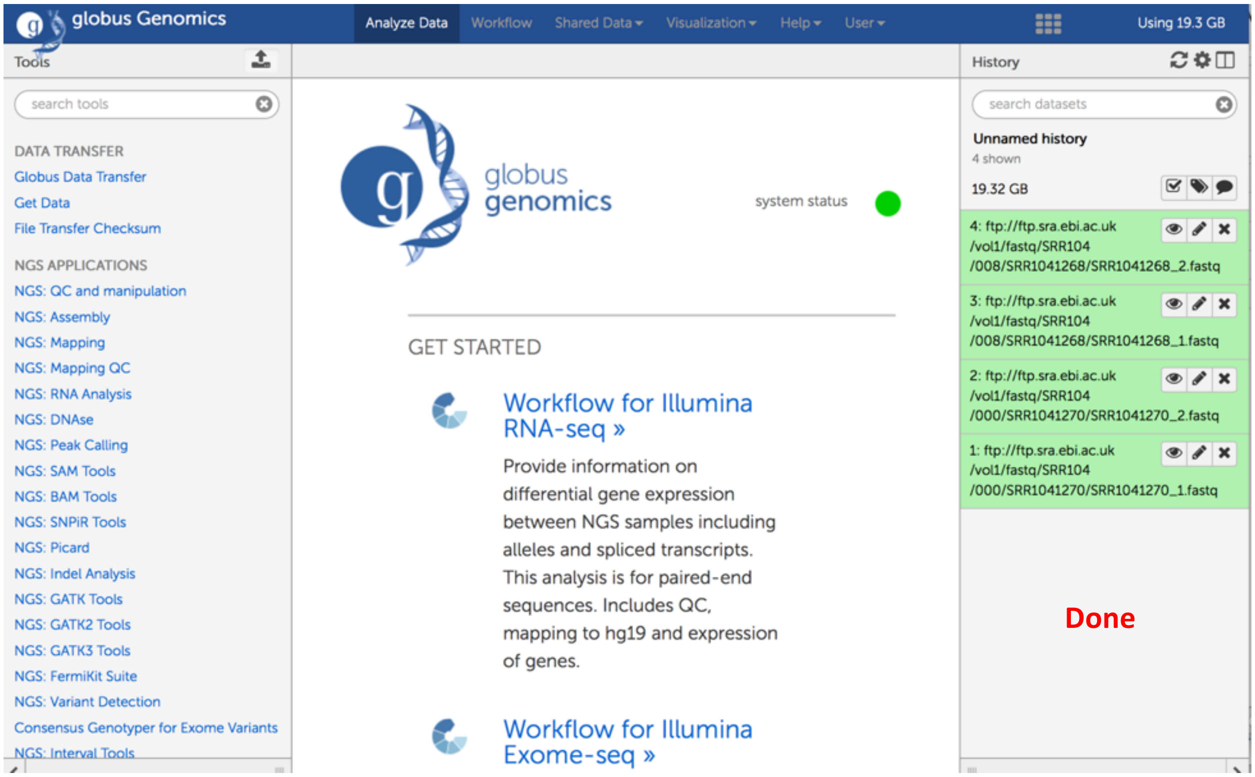
4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq.gz

3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz

2: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz

1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz

In Process



Provide information on differential gene expression between NGS samples including alleles and spliced transcripts. This analysis is for paired-end sequences. Includes QC, mapping to hg19 and expression of genes.

Unnamed history
4 shown
19.32 GB

4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq

3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq

2: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq

1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq

Done

Group assignments:

Group 1:

Plasmodium falciparum Asexual vs. Cultured sporozoites
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA230379

Samples:

Asexual samples:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz

Cultured sporozoite samples:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq.gz

Group 2:

Plasmodium falciparum Asexual vs. Salivary sporozoites
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA230379

Samples:

Asexual samples:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz

Salivary Sporozoites:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/006/SRR1041266/SRR1041266_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/006/SRR1041266/SRR1041266_2.fastq.gz

Group 3:

Plasmodium falciparum Cultured vs. Salivary sporozoites
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA230379

Samples:

Cultured sporozoite samples:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq.gz

Salivary Sporozoites:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/006/SRR1041266/SRR1041266_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/006/SRR1041266/SRR1041266_2.fastq.gz

Group 4:

Aspergillus nidulans FGSC4 VeA$^+$ WT vs. OSA knock outs
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA293709

Samples:

FGSC4 VeA$^+$ WT:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR218/001/SRR2180251/SRR2180251_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR218/001/SRR2180251/SRR2180251_2.fastq.gz

OSA knock outs:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR218/007/SRR2180257/SRR2180257_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR218/007/SRR2180257/SRR2180257_2.fastq.gz

Group 5:

Toxoplasma gondii WT vs. GRA17 knock outs
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA275621

Samples:

WT:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/001/SRR1805881/SRR1805881_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/001/SRR1805881/SRR1805881_2.fastq.gz

GRA17 knock outs:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/002/SRR1805882/SRR1805882_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/002/SRR1805882/SRR1805882_2.fastq.gz

Group 6:

Toxoplasma gondii WT vs. GRA17 knock outs
Project information: http://www.ebi.ac.uk/ena/data/view/PRJNA275621

Samples:

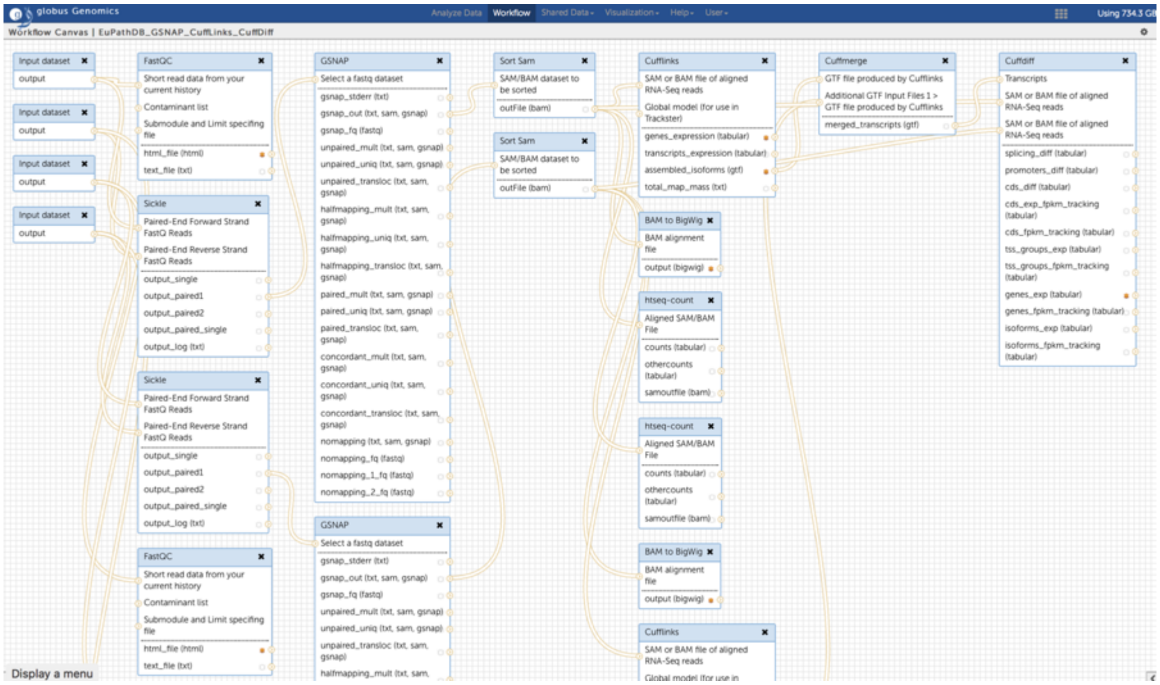
WT:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/001/SRR1805881/SRR1805881_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/001/SRR1805881/SRR1805881_2.fastq.gz

GRA23 knock outs:
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/003/SRR1805883/SRR1805883_1.fastq.gz
ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR180/003/SRR1805883/SRR1805883_2.fastq.gz

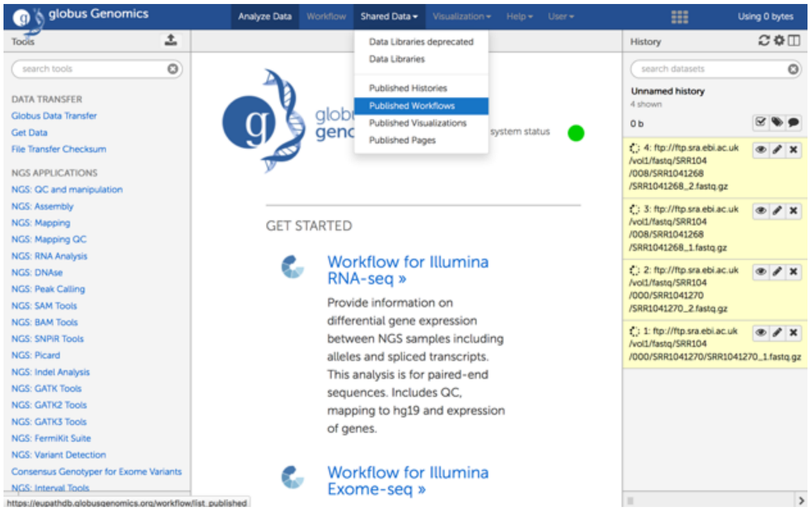
Section II: Running a workflow in Galaxy

You can create your own workflows in galaxy based on your needs. The tools in the left section can all be added and configured as steps in a workflow that can be run on appropriate datasets. For this exercise we will use a preconfigured workflow that does the following main things:

1. Analyzes the reads in your files and generates FASTQC reports.
2. Trims the reads based on their quality scores.
3. Aligns the reads to a reference genome using GSNAP and generates coverage plots.
4. Determines FPKM values for each sample and generates gene/transcript models.
5. Determines differential expression of genes between the samples.


globus Genomics
Analyze Data
Workflow
Shared Data
Visualization
Help
User
Using 734.3 GB
Workflow Canvas | EuPathDB_GSNAP_CuffLinks_CuffDiff
Input dataset
output
FastQC
Short read data from your current history
Contaminant list
Submodule and Limit specifing file
html_file (html)
text_file (txt)
Sickle
Paired-End Forward Strand FastQ Reads
Paired-End Reverse Strand FastQ Reads
output_single
output_paired1
output_paired2
output_paired_single
output_log (txt)
GSNAP
Select a fastq dataset
gsnap_stderr (txt)
gsnap_out (txt, sam, gsnap)
gsnap_fq (fastq)
unpaired_mult (txt, sam, gsnap)
unpaired_uniq (txt, sam, gsnap)
unpaired_transloc (txt, sam, gsnap)
halfmapping_mult (txt, sam, gsnap)
halfmapping_uniq (txt, sam, gsnap)
halfmapping_transloc (txt, sam, gsnap)
paired_mult (txt, sam, gsnap)
paired_uniq (txt, sam, gsnap)
paired_transloc (txt, sam, gsnap)
concordant_mult (txt, sam, gsnap)
concordant_uniq (txt, sam, gsnap)
concordant_transloc (txt, sam, gsnap)
nomapping (txt, sam, gsnap)
nomapping_fq (fastq)
nomapping_1_fq (fastq)
nomapping_2_fq (fastq)
Sort Sam
SAM/BAM dataset to be sorted
outFile (bam)
Cufflinks
SAM or BAM file of aligned RNA-Seq reads
Global model (for use in Trackster)
genes_expression (tabular)
transcripts_expression (tabular)
assembled_isoforms (gtf)
total_map_mass (txt)
BAM to BigWig
BAM alignment file
output (bigwig)
htseq-count
Aligned SAM/BAM File
counts (tabular)
othercounts (tabular)
samoutfile (bam)
Cuffmerge
GTF file produced by Cufflinks
Additional GTF Input Files 1 > GTF file produced by Cufflinks
merged_transcripts (gtf)
Cuffdiff
Transcripts
SAM or BAM file of aligned RNA-Seq reads
splicing_diff (tabular)
promoters_diff (tabular)
cds_diff (tabular)
cds_exp_fpkm_tracking (tabular)
cds_fpkm_tracking (tabular)
tss_groups_exp (tabular)
tss_groups_fpkm_tracking (tabular)
genes_exp (tabular)
genes_fpkm_tracking (tabular)
isoforms_exp (tabular)
isoforms_fpkm_tracking (tabular)
Display a menu

Step 1: Import the workflow called "EuPathDB_GSNAP_CuffLinks_CuffDiff" – click on the shared data menu item and select "Published Workflows" from the menu.



Provide information on differential gene expression between NGS samples including alleles and spliced transcripts. This analysis is for paired-end sequences. Includes QC, mapping to hg19 and expression of genes.

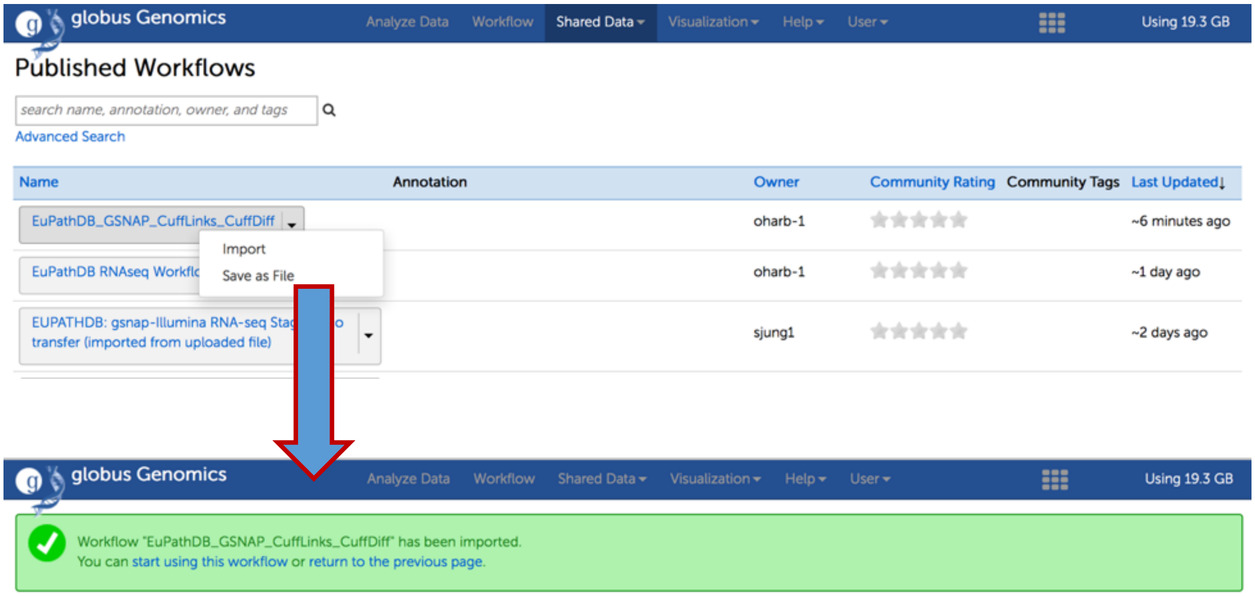
4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq.gz

3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq.gz

2: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq.gz

1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq.gz

Step 2: Click on the arrow next to the appropriate workflow and select import.

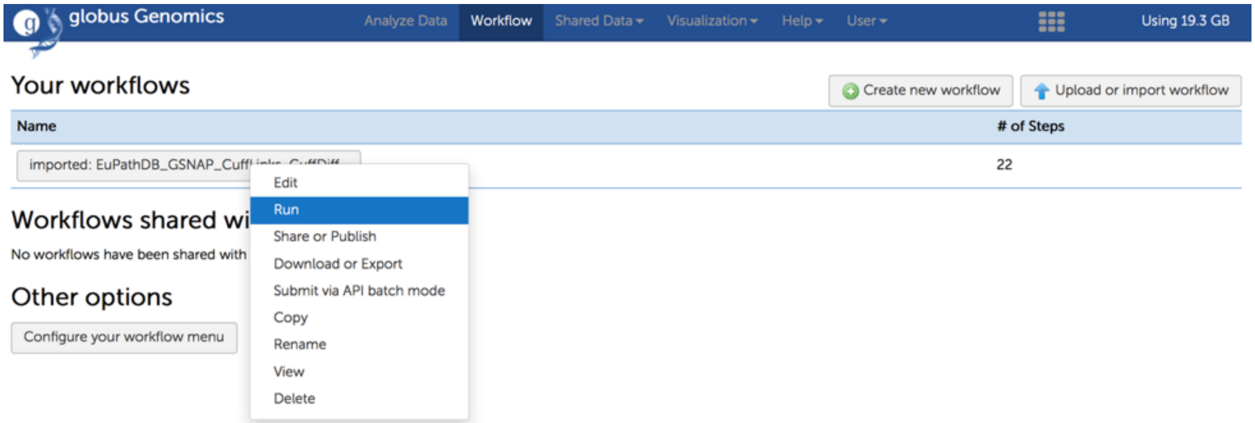


Published Workflows

Name	Annotation	Owner	Community Rating	Community Tags	Last Updated↓
EuPathDB_GSNAP_CuffLinks_CuffDiff		oharb-1	☆☆☆☆☆		~6 minutes ago
EuPathDB RNAseq Workflo		oharb-1	☆☆☆☆☆		~1 day ago
EUPATHDB: gsnap-Illumina RNA-seq Stag o transfer (imported from uploaded file)		sjung1	☆☆☆☆☆		~2 days ago

✓ Workflow "EuPathDB_GSNAP_CuffLinks_CuffDiff" has been imported.
You can start using this workflow or return to the previous page.

Step 3: Click on "Workflow" in the menu at the top of the page. On the next page click on the arrow next to your imported workflow and select the "Run" option.



Your workflows

Name	# of Steps
imported: EuPathDB_GSNAP_Cufflinks_CuffDiff	22

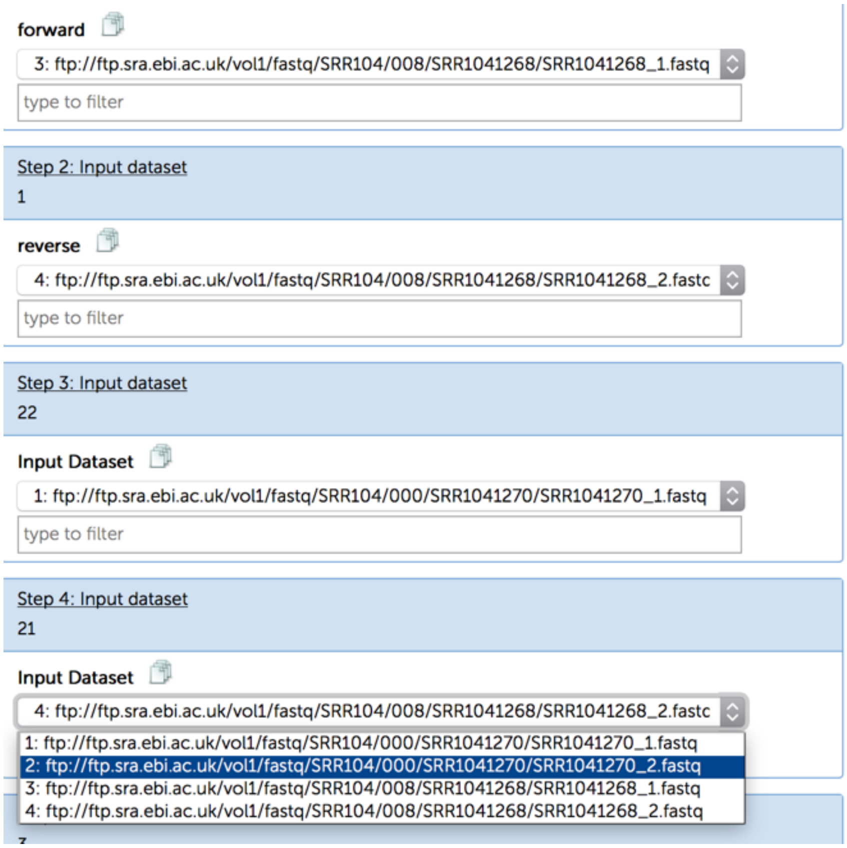
Workflows shared wi

No workflows have been shared with

Other options

Step 4: Configure your workflow – there are multiple steps in the workflow but you do not need to configure all of them. For the purpose of this exercise you will need to configure the following:

a. Select the input datasets. These are the fastq files you imported from the sequence archive. Workflow steps 1-4 allow you to select the datasets. Be sure you match the correct forward and reverse files. The should end in the same SRR number with a .1 or .2 at the end.



forward

3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq

type to filter

Step 2: Input dataset

1

reverse

4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastc

type to filter

Step 3: Input dataset

22

Input Dataset

1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq

type to filter

Step 4: Input dataset

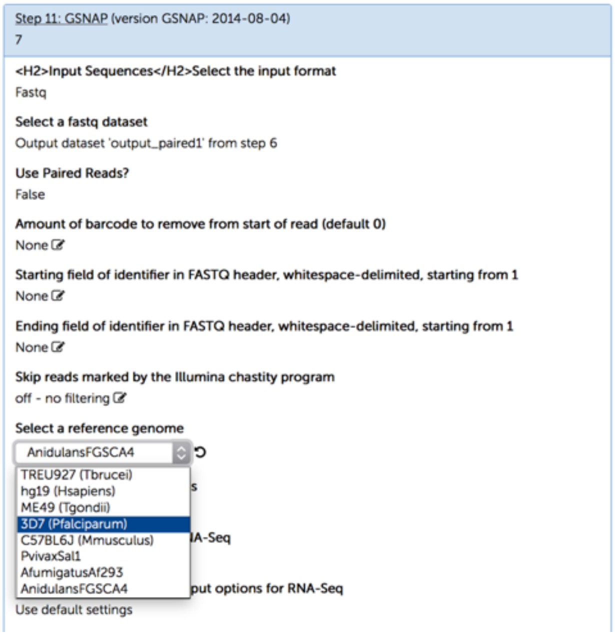
21

Input Dataset

4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastc

1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq
2: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_2.fastq
3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq
4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq

b. Scroll down to steps 11 and 12 (GSNAP). Click on the name of the step to open up the parameters. Select the correct reference organism in each of the steps.



Step 11: GSNAP (version GSNAP: 2014-08-04)
7

<H2>Input Sequences</H2>Select the input format
Fastq

Select a fastq dataset
Output dataset 'output_paired1' from step 6

Use Paired Reads?
False

Amount of barcode to remove from start of read (default 0)
None

Starting field of identifier in FASTQ header, whitespace-delimited, starting from 1
None

Ending field of identifier in FASTQ header, whitespace-delimited, starting from 1
None

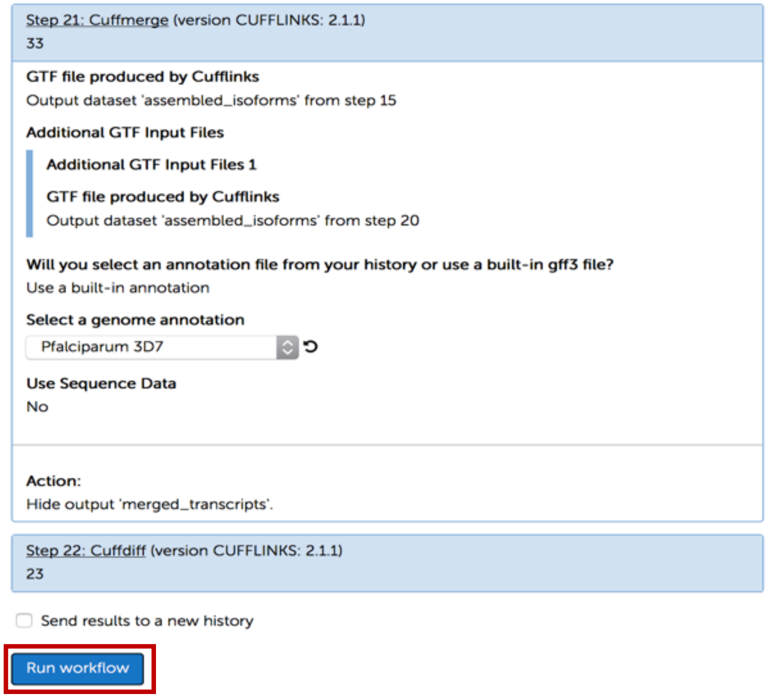
Skip reads marked by the Illumina chastity program
off - no filtering

Select a reference genome
AnidulansFGSCA4

put options for RNA-Seq
Use default settings

c. Scroll down to step 15 (Cufflinks), 17, 18 (htseq), 20 (Cufflinks) and 21 (Cuffmerge) and select the correct reference organism.

d. Click on "Run Workflow"



Step 21: Cuffmerge (version CUFFLINKS: 2.1.1)
33

GTF file produced by Cufflinks
Output dataset 'assembled_isoforms' from step 15

Additional GTF Input Files

Additional GTF Input Files 1

GTF file produced by Cufflinks
Output dataset 'assembled_isoforms' from step 20

Will you select an annotation file from your history or use a built-in gff3 file?
Use a built-in annotation

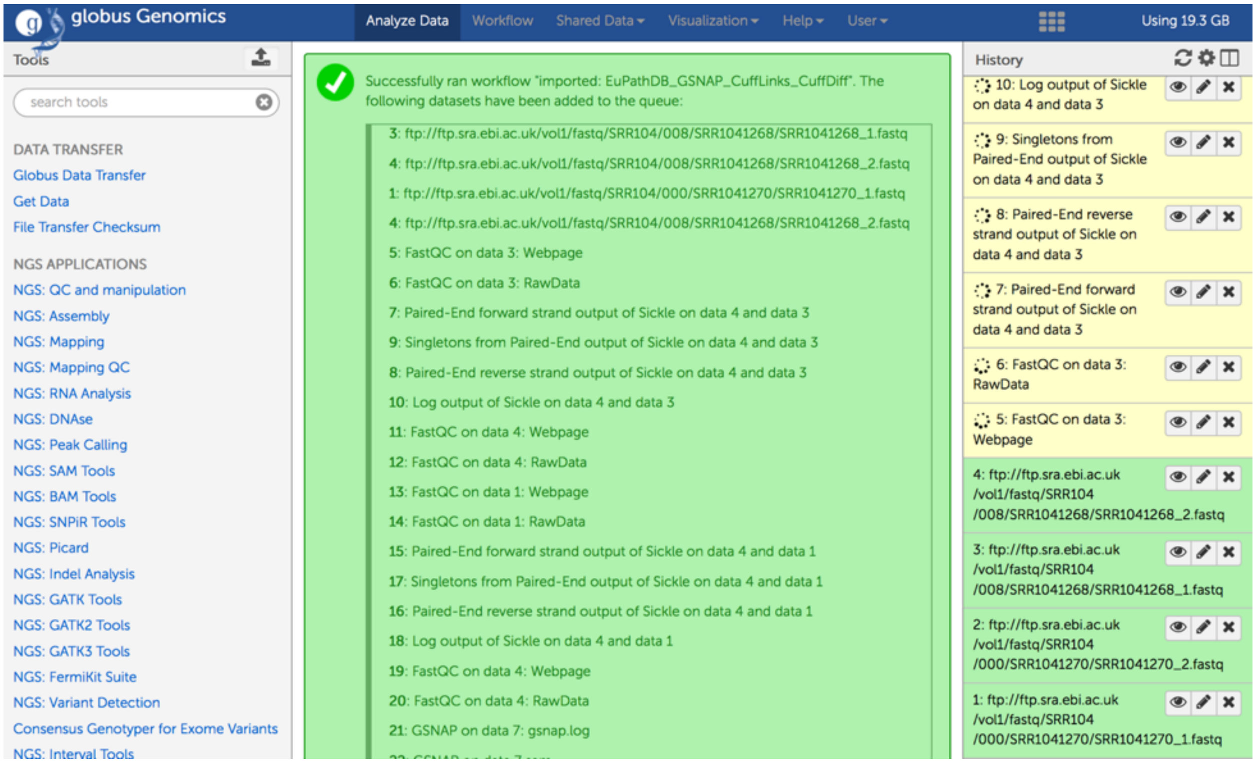
Select a genome annotation
Pfalciparum 3D7

Use Sequence Data
No

Action:
Hide output 'merged_transcripts'.

Step 22: Cuffdiff (version CUFFLINKS: 2.1.1)
23

□ Send results to a new history



Successfully ran workflow "imported: EuPathDB_GSNAP_CuffLinks_CuffDiff". The following datasets have been added to the queue:

3: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_1.fastq
4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq
1: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/000/SRR1041270/SRR1041270_1.fastq
4: ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR104/008/SRR1041268/SRR1041268_2.fastq
5: FastQC on data 3: Webpage
6: FastQC on data 3: RawData
7: Paired-End forward strand output of Sickle on data 4 and data 3
9: Singletons from Paired-End output of Sickle on data 4 and data 3
8: Paired-End reverse strand output of Sickle on data 4 and data 3
10: Log output of Sickle on data 4 and data 3
11: FastQC on data 4: Webpage
12: FastQC on data 4: RawData
13: FastQC on data 1: Webpage
14: FastQC on data 1: RawData
15: Paired-End forward strand output of Sickle on data 4 and data 1
17: Singletons from Paired-End output of Sickle on data 4 and data 1
16: Paired-End reverse strand output of Sickle on data 4 and data 1
18: Log output of Sickle on data 4 and data 1
19: FastQC on data 4: Webpage
20: FastQC on data 4: RawData
21: GSNAP on data 7: gsnap.log

10: Log output of Sickle on data 4 and data 3

9: Singletons from Paired-End output of Sickle on data 4 and data 3

8: Paired-End reverse strand output of Sickle on data 4 and data 3

7: Paired-End forward strand output of Sickle on data 4 and data 3

6: FastQC on data 3: RawData

5: FastQC on data 3: Webpage

4: ftp://ftp.sra.ebi.ac.uk /vol1/fastq/SRR104 /008/SRR1041268/SRR1041268_2.fastq

3: ftp://ftp.sra.ebi.ac.uk /vol1/fastq/SRR104 /008/SRR1041268/SRR1041268_1.fastq

2: ftp://ftp.sra.ebi.ac.uk /vol1/fastq/SRR104 /000/SRR1041270/SRR1041270_2.fastq

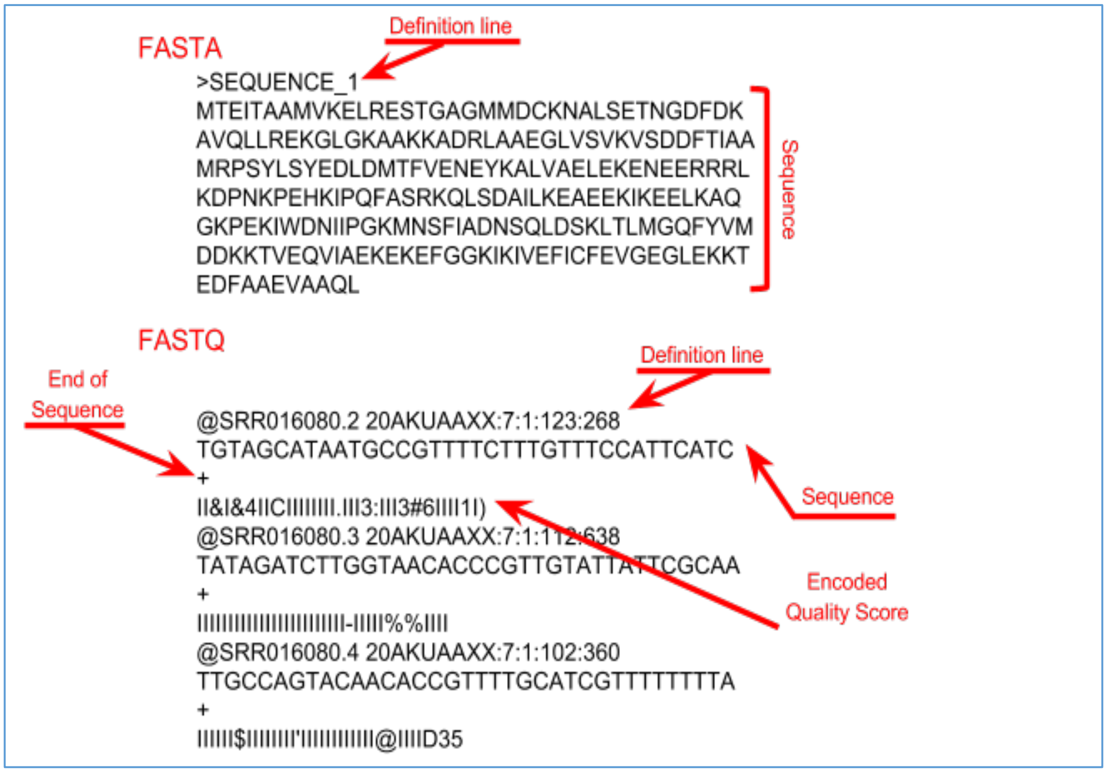
1: ftp://ftp.sra.ebi.ac.uk /vol1/fastq/SRR104 /000/SRR1041270/SRR1041270_1.fastq

The steps will start running in the history section on the right. Grey means they are waiting to start. Yellow means they are running. Green means they have completed. Red means there was an error in the step.

Appendix:

FASTQ file are text files (similar to FASTA) that include sequence quality information and details in addition to the sequence (ie. name, quality scores, sequencing machine ID, lane number etc.). FASTQ files are large and as a result not all sequencing repositories will store this format. However, tools are available to convert, for example, NCBI's SRA format to FASTQ. Sequence data is housed in three repositories that are synchronized on a regular basis.

- The sequence read archive at GenBank
- The European Nucleotide Archive at EMBL
- The DNA data bank of Japan



```
>SEQUENCE_1
MTEITAAMVKELRESTGAGMMDCKNALSETNGDFDK
AVQLLREKGLGKAAKKADRLAAEGLVSVKVSDDFTIAA
MRPSYLSYEDLDMTFVENEYKALVAELEKENEERRRL
KDPNKPEHKIPQFASRKQLSDAILKEAEEKIKEELKAQ
GKPEKIWDNIIPGKMNSFIADNSQLDSKLTLMGQFYVM
DDKKTVEQVIAEKEKEFGGKIKIVEFICFEVGEGLEKKT
EDFAAEVAAQL
```

```
@SRR016080.2 20AKUAAXX:7:1:123:268
TGTAGCATAATGCCGTTTTCTTTGTTTCCATTCATC
+
II&I&4IICIIIIIIII.III3:III3#6IIII1I)
@SRR016080.3 20AKUAAXX:7:1:112:638
TATAGATCTTGGTAACACCCGTTGTATTATTCGCAA
+
IIIIIIIIIIIIIIIIIIIIIIII-IIIII%%IIII
@SRR016080.4 20AKUAAXX:7:1:102:360
TTGCCAGTACAACACCGTTTTGCATCGTTTTTTTTA
+
IIIIII$IIIIIIII'IIIIIIIIIII@IIIID35
```