Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) V.3

3

Mar 08, 2022

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dx.doi.org/10.17504/protocols.io.b5u2q6ye

GenomeTrakr
Tech. support email: **genomeTrakr@fda.hhs.gov**

Jasmine Amirzadegan

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PURPOSE:

Step-by-step instructions for checking sequence quality for SARS-CoV-2 wastewater samples using **SSQuAWK2: S**ARS - CoV - 2 **S**equence **Qu**ality **A**ssurance **W**orkflow and **K**ontraption, version **2**. The SSQuAWK2 workflow, implemented in a custom Galaxy instance, will produce quality assessments for raw reads (Illumina MiSeq paired-end fastq files).

SCOPE: This protocol covers the following tasks:

1. Set up an account in GalaxyTrakr
2. Create a new history
3. Upload data and reference files
4. Execute the SSQuAWK2 workflow
5. Interpret the results

Protocol and SSQuAWK workflow version history:
Protocol V1, SSQuAWK version 1: Basic protocol steps with screenshots
Protocol V2, SSQuAWK version 1: Addition of a detailed 12 minute video tutorial
Protocol V3, SSQuAWK version 2: Addition of 5 new genome mapping metrics

DOI

dx.doi.org/10.17504/protocols.io.b5u2q6ye

https://galaxytrakr.org

Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey 2022. Wastewater QC workflow in GalaxyTrakr (SSQuAWK2). **protocols.io**
https://dx.doi.org/10.17504/protocols.io.b5u2q6ye
Jasmine Amirzadegan

WGS, Quality Control, GalaxyTrakr, GenomeTrakr, microbial pathogen survielliance

protocol ,

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Mar 08, 2022

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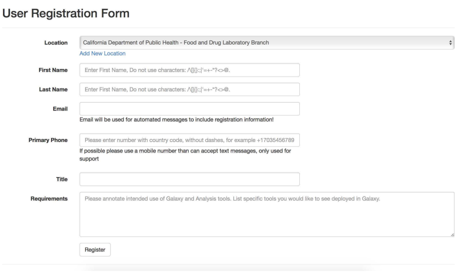
Account set up

1 1. **Create a GalaxyTrakr account here: https://account.galaxytrakr.org/Account/Register**



User Registration Form

Location: California Department of Public Health - Food and Drug Laboratory Branch

Add New Location

First Name: Enter First Name, Do not use characters: /\[]{}:;|'=+-*?<>@.

Last Name: Enter First Name, Do not use characters: /\[]{}:;|'=+-*?<>@.

Email:

Email will be used for automated messages to include registration information!

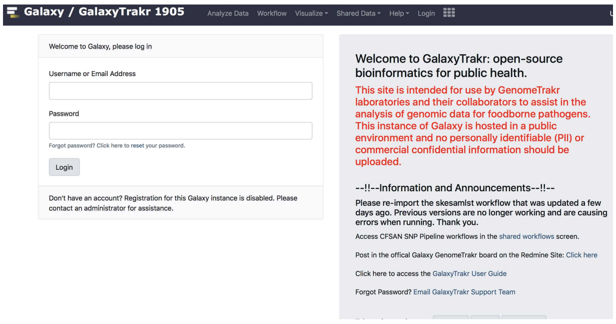
Primary Phone: Please enter number with country code, without dashes, for example +17035456789

If possible please use a mobile number than can accept text messages, only used for support

Title:

Requirements: Please annotate intended use of Galaxy and Analysis tools. List specific tools you would like to see deployed in Galaxy.

1.1 Log into your GalaxyTrakr account: https://galaxytrakr.org



Welcome to Galaxy, please log in

Username or Email Address

Password

Forgot password? Click here to reset your password.

Don't have an account? Registration for this Galaxy instance is disabled. Please contact an administrator for assistance.

Welcome to GalaxyTrakr: open-source bioinformatics for public health.

This site is intended for use by GenomeTrakr laboratories and their collaborators to assist in the analysis of genomic data for foodborne pathogens. This instance of Galaxy is hosted in a public environment and no personally identifiable (PII) or commercial confidential information should be uploaded.

--!!--Information and Announcements--!!--

Please re-import the skesamlst workflow that was updated a few days ago. Previous versions are no longer working and are causing errors when running. Thank you.

Access CFSAN SNP Pipeline workflows in the shared workflows screen.

Post in the offical Galaxy GenomeTrakr board on the Redmine Site: Click here

Click here to access the GalaxyTrakr User Guide

Forgot Password? Email GalaxyTrakr Support Team

Create a new history

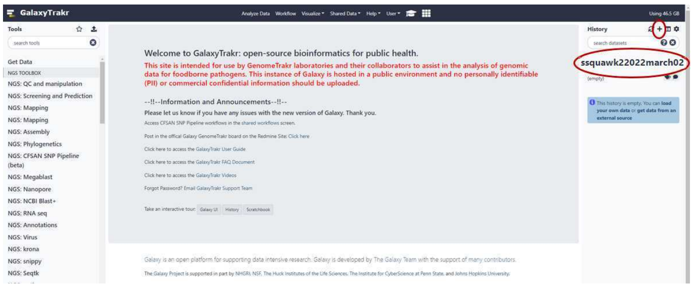
2 **Create a new history.**

We recommend creating a new history for each new MiSeq sequence set with details and date in the history name.

Save your SSQuAWK2 output here with any other relevant analyses.

After all the analysis output from this run is saved to your internal data network or computer, older history's should be purged/deleted so as not to occupy the limited storage space in your account. In some cases it may be useful to save, for a limited time, multiple histories or to run analyses concurrently in multiple histories. In these cases you need to pay attention to your % usage bar (shows % used of allocated storage space) in the upper right corner of the GalaxyTrakr page. If you need additional space you can contact galaxytrakrsupport@fda.hhs.gov and request additional storage.

2.1 Create a new history with the "+" symbol in the upper right hand corner. Name your history and press "enter" on your keyboard to save the name.



Welcome to GalaxyTrakr: open-source bioinformatics for public health.

This site is intended for use by GenomeTrakr laboratories and their collaborators to assist in the analysis of genomic data for foodborne pathogens. This instance of Galaxy is hosted in a public environment and no personally identifiable (PII) or commercial confidential information should be uploaded.

--!!--Information and Announcements--!!--

Please let us know if you have any issues with the new version of Galaxy. Thank you.

Access CFSAN SNP Pipeline workflows in the shared workflows screen.

Post in the offical Galaxy GenomeTrakr board on the Redmine Site: Click here

Click here to access the GalaxyTrakr User Guide

Click here to access the GalaxyTrakr FAQ Document

Click here to access the GalaxyTrakr Videos

Forgot Password? Email GalaxyTrakr Support Team

Take an interactive tour:

This history is empty. You can load your own data or get data from an external source

Galaxy is an open platform for supporting data intensive research. Galaxy is developed by The Galaxy Team with the support of many contributors.

The Galaxy Project is supported in part by NHGRI, NSF, The Huck Institutes of the Life Sciences, The Institute for CyberScience at Penn State, and Johns Hopkins University.

Upload sequence data

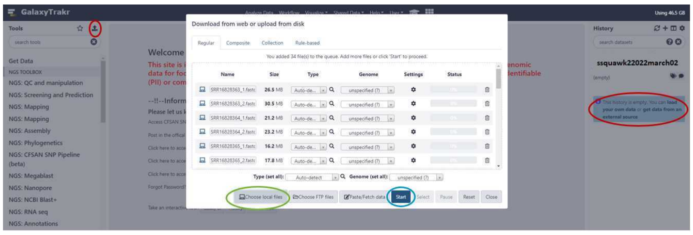
3 **This section will describe the process for uploading raw fastq files into your active History panel.** After the files have been uploaded they will stay in your account until they are deleted.

3.1 Upload sequence data to your history, using either of the two options circled in red below.

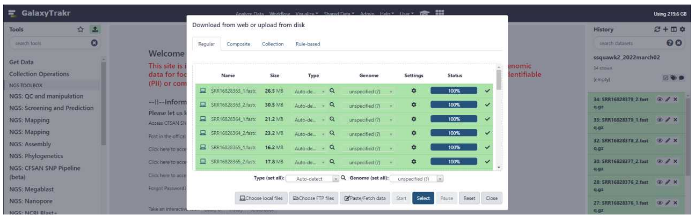
A window will appear in the middle of your screen. This is where you select your files using the "Choose local files" button at the bottom of the window. The "Choose local files" button is circled in green. These fastq files should be paired (two per sample).

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye

After you've selected your files, press "Start" to initiate your data upload to GalaxyTrakr. The "Start" button is circled in blue.

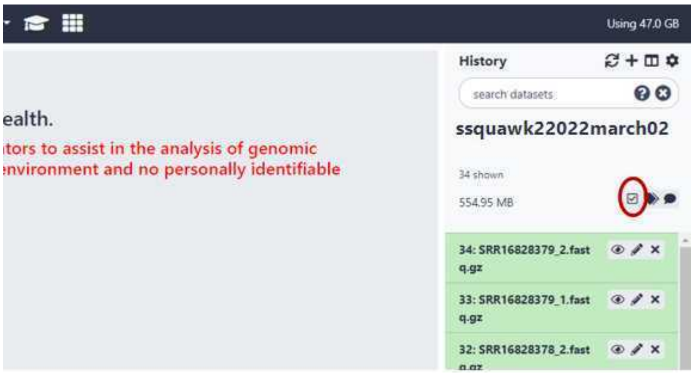


3.2 As the file uploads complete, each row will turn green. If samples are shown with yellow background, then are still uploading.



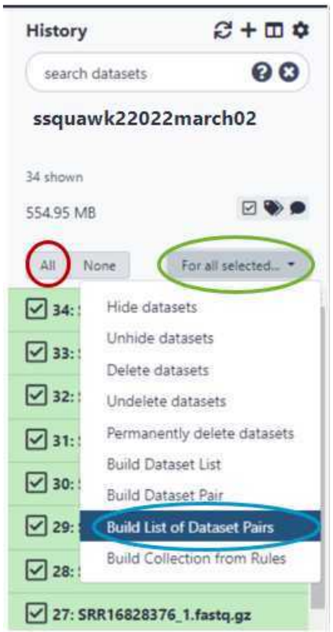
3.3 You have just upload a set of forward and reverse reads. For further analysis these files need to be paired properly so the platform knows which R1 and R2 files go with each sample. GalaxyTrakr does this by creating a **List of Dataset Pairs.**

Within your newly created History panel, click the "check box," then select all the files you just uploaded by clicking "All" or by individually selecting the ones you want to pair.


Using 47.0 GB
History
search datasets
ssquawk22022march02
34 shown
554.95 MB
34: SRR16828379_2.fastq.gz
33: SRR16828379_1.fastq.gz
32: SRR16828378_2.fastq.gz
ealth.
tors to assist in the analysis of genomic
nvironment and no personally identifiable

3.4 Check all the files belonging to a pair. In this example, all the files belong to a pair, so I will use the "All" button (circled in red).

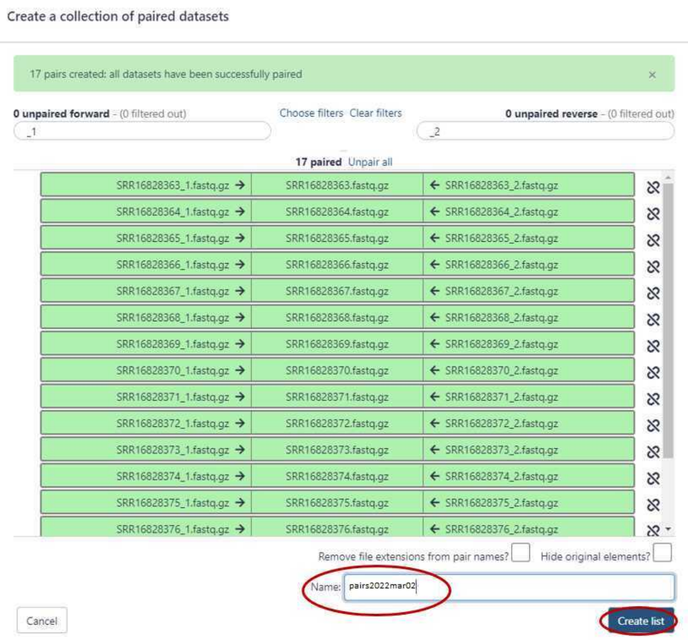
Then, use the "For all selected..." dropdown (circled in green), and click on "Build List of Dataset Pairs" (circled in blue).



3.5 GalaxyTrakr will automatically pair the files, but it's good to double check.

Paired reads will pair in the middle column and turn green.

If everything looks good, then choose a name for your pairs (circled red) and "Create List" (also circled red).


Create a collection of paired datasets

17 pairs created: all datasets have been successfully paired

0 unpaired forward - (0 filtered out) _1

0 unpaired reverse - (0 filtered out) _2

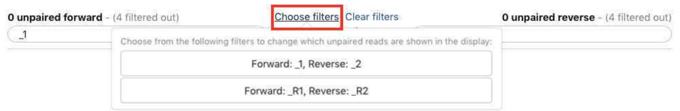
17 paired

SRR16828363_1.fastq.gz →	SRR16828363.fastq.gz	← SRR16828363_2.fastq.gz
SRR16828364_1.fastq.gz →	SRR16828364.fastq.gz	← SRR16828364_2.fastq.gz
SRR16828365_1.fastq.gz →	SRR16828365.fastq.gz	← SRR16828365_2.fastq.gz
SRR16828366_1.fastq.gz →	SRR16828366.fastq.gz	← SRR16828366_2.fastq.gz
SRR16828367_1.fastq.gz →	SRR16828367.fastq.gz	← SRR16828367_2.fastq.gz
SRR16828368_1.fastq.gz →	SRR16828368.fastq.gz	← SRR16828368_2.fastq.gz
SRR16828369_1.fastq.gz →	SRR16828369.fastq.gz	← SRR16828369_2.fastq.gz
SRR16828370_1.fastq.gz →	SRR16828370.fastq.gz	← SRR16828370_2.fastq.gz
SRR16828371_1.fastq.gz →	SRR16828371.fastq.gz	← SRR16828371_2.fastq.gz
SRR16828372_1.fastq.gz →	SRR16828372.fastq.gz	← SRR16828372_2.fastq.gz
SRR16828373_1.fastq.gz →	SRR16828373.fastq.gz	← SRR16828373_2.fastq.gz
SRR16828374_1.fastq.gz →	SRR16828374.fastq.gz	← SRR16828374_2.fastq.gz
SRR16828375_1.fastq.gz →	SRR16828375.fastq.gz	← SRR16828375_2.fastq.gz
SRR16828376_1.fastq.gz →	SRR16828376.fastq.gz	← SRR16828376_2.fastq.gz

Remove file extensions from pair names? □ Hide original elements? □

Name: pairs2022mar02

Alternatively, instead of auto-pairing you can click "choose filters" and select the appropriate filter for the pairing:

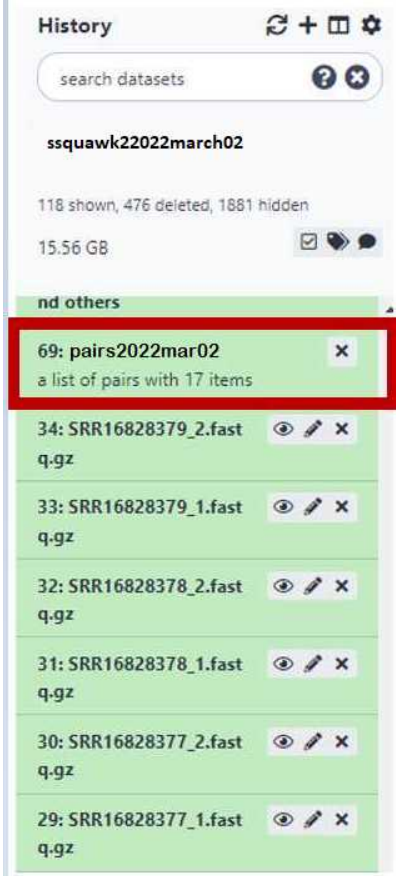

0 unpaired forward - (4 filtered out) _1

0 unpaired reverse - (4 filtered out)

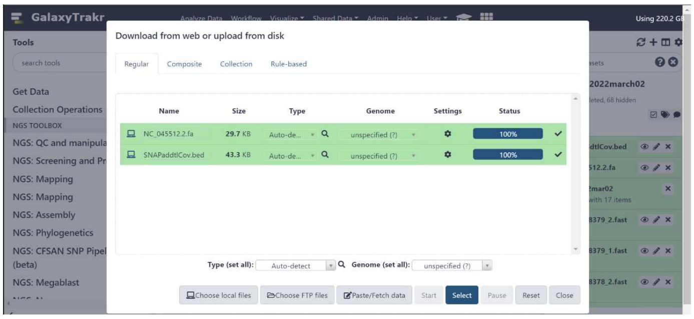
Choose from the following filters to change which unpaired reads are shown in the display:

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye

3.6 This paired dataset will now be available for analysis in your history panel. You can run multiple analyses on the same dataset in a history rather than upload the same sequence data to a new history to perform additional analyses. This will help you use your allocated storage space efficiently.


History
search datasets
ssquawk22022march02
118 shown, 476 deleted, 1881 hidden
15.56 GB
nd others
69: pairs2022mar02
a list of pairs with 17 items
34: SRR16828379_2.fastq.gz
33: SRR16828379_1.fastq.gz
32: SRR16828378_2.fastq.gz
31: SRR16828378_1.fastq.gz
30: SRR16828377_2.fastq.gz
29: SRR16828377_1.fastq.gz

4 To the existing history, also upload (1) the **provided reference.fasta file** and (1) a primer.bed file.



Download from web or upload from disk

Name	Size	Type	Genome	Settings	Status
NC_045512.2.fa	29.7 KB	Auto-de...	unspecified (?)	⚙	100% ✓
SNAPaddtlCov.bed	43.3 KB	Auto-de...	unspecified (?)	⚙	100% ✓

Type (set all): Auto-detect Genome (set all): unspecified (?)

4.1 **SSQuAWK2 is only compatible with the 22903 nt reference genome file obtained from NCBI 'NC_045512.2'. It is provided here for your convenience:** NC_045512.2.fa

4.2 **The primer.bed file should correspond to the SARS - CoV - 2 enrichment primer panel kit used.**

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye

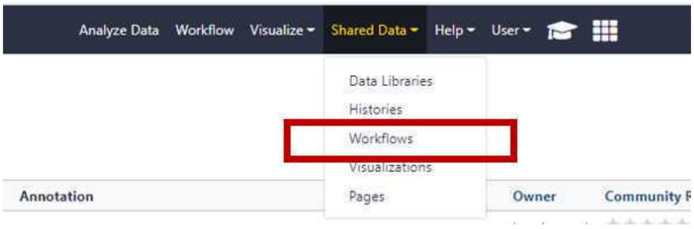
QIAseqDIRECT.bed	QIAseq Direct kit
SNAPStd.bed	SNAP standard kit
SNAPaddtlCov.bed	SNAP additional coverage kit
varskipShort.bed	NEB VarSkip Short (version 1) kit
VSSv2.primer.bed	NEB VarSkip Short (version 2) kit
ARTICv4.bed	ARTIC v4 primer schemes

Run the SSQuAWK workflow

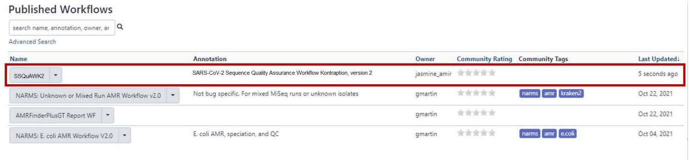
5 **Add the SSQuAWK2* workflow to your own "workflows" panel.** You only have to do this step once for each new workflow you need.

***SSQuAWK2: S**ARS - CoV - 2 **S**equence **Qu**ality **A**ssurance **W**orkflow **K**ontraption, version **2**

5.1 Navigate to the “Shared Data" drop down and choose workflows



Then, from the SSQuaWK2 drop down menu, select import.

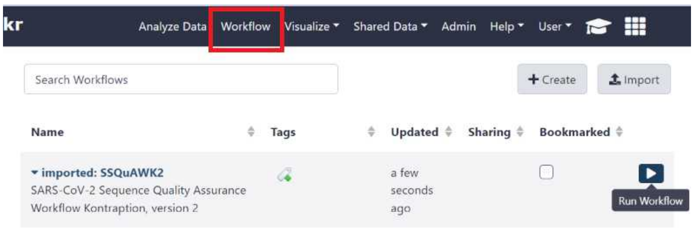

Published Workflows

search name, annotation, owner, ar

Advanced Search

Name	Annotation	Owner	Community Rating	Community Tags	Last Updated↓
SSQuAWK2	SARS-CoV-2 Sequence Quality Assurance Workflow Kontraption, version 2	jasmine_amir	☆☆☆☆☆		5 seconds ago
NARMS: Unknown or Mixed Run AMR Workflow v2.0	Not bug specific. For mixed MiSeq runs or unknown isolates	gmartin	☆☆☆☆☆	narms amr kraken2	Oct 22, 2021
AMRFinderPlusGT Report WF		gmartin	☆☆☆☆☆		Oct 22, 2021
NARMS: E. coli AMR Workflow V2.0	E. coli AMR, speciation, and QC	gmartin	☆☆☆☆☆	narms amr e.coli	Oct 04, 2021

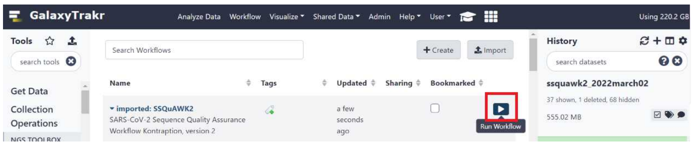
5.2 Navigate to the "Workflow" tab in the top ribbon (boxed in red). The workflow will be imported there.



Search Workflows

Name	Tags	Updated	Sharing	Bookmarked
imported: SSQuAWK2 SARS-CoV-2 Sequence Quality Assurance Workflow Kontraption, version 2		a few seconds ago		□

5.3 To use the workflow, press the 'play' button (boxed in red) on the right



imported: SSQuAWK2
SARS-CoV-2 Sequence Quality Assurance Workflow Kontraption, version 2
a few seconds ago

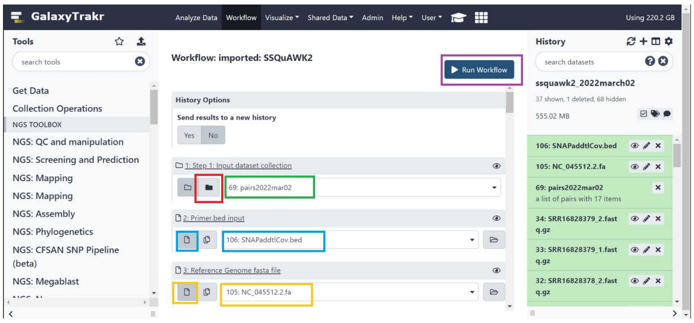
ssquawk2_2022march02
37 shown, 1 deleted, 68 hidden
555.02 MB

5.4 Select the paired list you created earlier by selecting the folder icon (boxed in red), and then the list of pairs (boxed in green).

Boxed in blue: Select the bed file from your history.

Boxed in gold: Select the reference fasta file from your history.

Click Run Workflow (boxed in purple).



Workflow: imported: SSQuAWK2

History Options

Send results to a new history: Yes No

1: Step 1: Input dataset collection: 69: pairs2022mar02

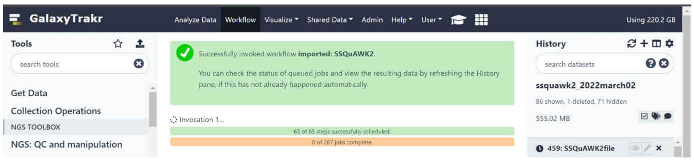
2: Primer.bed input: 106: SNAPaddtlCov.bed

3: Reference Genome fasta file: 105: NC_045512.2.fa

ssquawk2_2022march02
37 shown, 1 deleted, 68 hidden
555.02 MB

106: SNAPaddtlCov.bed
105: NC_045512.2.fa
69: pairs2022mar02
a list of pairs with 17 items
34: SRR16828379_2.fastq.gz
33: SRR16828379_1.fastq.gz
32: SRR16828378_2.fastq.gz

Running the workflow can take some time depending on the number of samples you are analyzing. Once GalaxyTrakr adds the workflow invocation to the queue, you can choose to log out of GalaxyTrakr and log back in at a later time to see if the job is completed.



Successfully invoked workflow **imported: SSQuAWK2**.

You can check the status of queued jobs and view the resulting data by refreshing the History pane, if this has not already happened automatically.

Invocation 1...

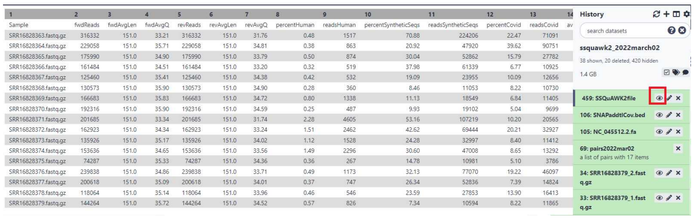
65 of 65 steps successfully scheduled.

0 of 287 jobs complete.

ssquawk2_2022march02
86 shown, 1 deleted, 71 hidden
555.02 MB

459: SSQuAWK2file

5.5 Upon completion of the pipeline, the **SSQuAWK2file** will be green. Click on the “Eye” icon to view the output in the GalaxyTrakr window.



1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample	fwdReads	fwdAvgLen	fwdAvgQ	revReads	revAvgLen	revAvgQ	percentHuman	readsHuman	percentSyntheticSeqs	readsSyntheticSeqs	percentCovid	readsCovid	av
SRR16828363.fastq.gz	316332	151.0	33.21	316332	151.0	31.76	0.48	1517	70.88	224206	22.47	71091	
SRR16828364.fastq.gz	229058	151.0	35.71	229058	151.0	34.81	0.38	863	20.92	47920	39.62	90751	
SRR16828365.fastq.gz	175990	151.0	34.90	175990	151.0	33.79	0.50	874	30.04	52862	15.79	27782	
SRR16828366.fastq.gz	161484	151.0	34.51	161484	151.0	33.20	0.32	519	37.98	61339	6.77	10925	
SRR16828367.fastq.gz	125460	151.0	35.41	125460	151.0	34.38	0.42	532	19.09	23955	10.09	12656	
SRR16828368.fastq.gz	130573	151.0	35.90	130573	151.0	34.90	0.28	360	8.46	11053	8.22	10730	
SRR16828369.fastq.gz	166683	151.0	35.83	166683	151.0	34.72	0.80	1338	11.13	18549	6.84	11405	
SRR16828370.fastq.gz	192316	151.0	35.90	192316	151.0	34.59	0.25	487	9.93	19102	5.04	9699	
SRR16828371.fastq.gz	201685	151.0	33.34	201685	151.0	31.74	2.28	4605	53.16	107219	10.20	20565	
SRR16828372.fastq.gz	162923	151.0	34.34	162923	151.0	33.24	1.51	2462	42.62	69444	20.21	32927	
SRR16828373.fastq.gz	135926	151.0	35.17	135926	151.0	34.02	1.12	1528	24.28	32997	8.40	11412	
SRR16828374.fastq.gz	153636	151.0	34.65	153636	151.0	33.56	1.49	2296	30.60	47008	8.65	13292	
SRR16828375.fastq.gz	74287	151.0	35.33	74287	151.0	34.36	0.36	267	14.78	10981	5.10	3786	
SRR16828376.fastq.gz	239838	151.0	34.86	239838	151.0	33.71	0.49	1173	32.13	77070	19.22	46097	
SRR16828377.fastq.gz	200618	151.0	35.09	200618	151.0	34.01	0.37	747	26.34	52836	7.39	14824	
SRR16828378.fastq.gz	118064	151.0	35.14	118064	151.0	33.96	0.46	546	23.59	27853	13.90	16413	
SRR16828379.fastq.gz	144264	151.0	35.72	144264	151.0	34.52	0.57	826	7.34	10594	8.22	11865	

ssquawk2_2022march02
38 shown, 20 deleted, 420 hidden
1.4 GB

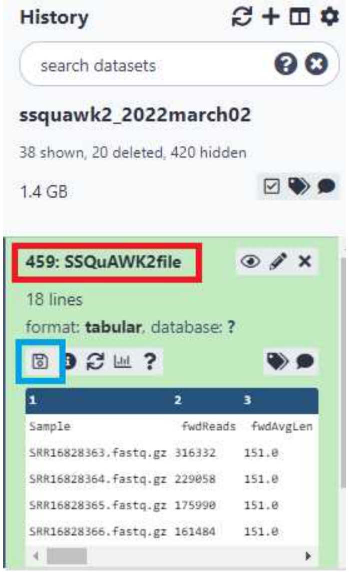
459: SSQuAWK2file
106: SNAPaddtlCov.bed
105: NC_045512.2.fa
69: pairs2022mar02
a list of pairs with 17 items
34: SRR16828379_2.fastq.gz
33: SRR16828379_1.fastq.gz

Interpret the results

6 Download and interpret the results:

6.1 Click "**SSQuAWK2file**” (boxed in red) and then the floppy disc save icon (boxed in blue). The tabular file can be opened in a text reader or converted to a format that can be opened in Excel.

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye



ssquawk2_2022march02

38 shown, 20 deleted, 420 hidden

1.4 GB

459: SSQuAWK2file

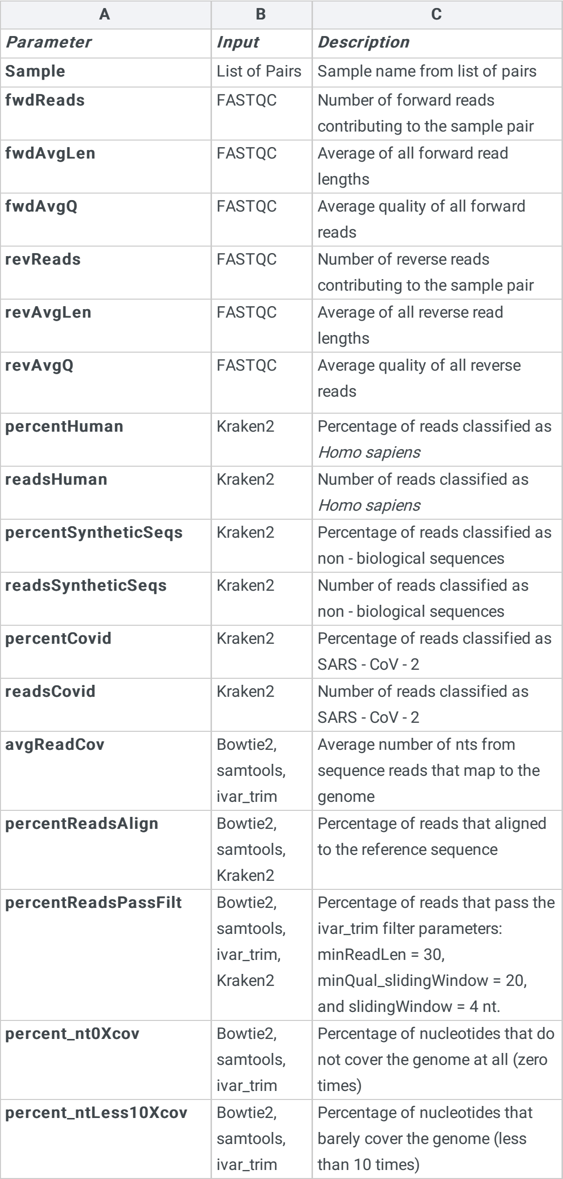
18 lines

format: **tabular**, database: ?

1	2	3
Sample	fwdReads	fwdAvgLen
SRR16828363.fastq.gz	316332	151.0
SRR16828364.fastq.gz	229058	151.0
SRR16828365.fastq.gz	175990	151.0
SRR16828366.fastq.gz	161484	151.0

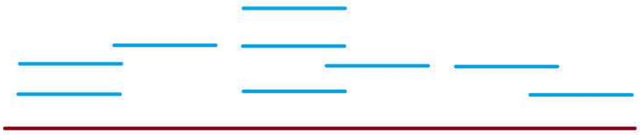
6.2 The SSQuAWK2 output file includes the following metrics:

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye



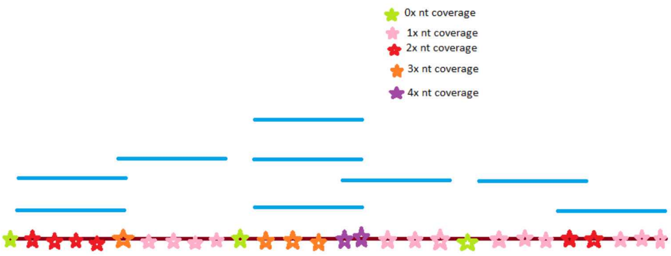
A	B	C
Parameter	***Input***	***Description***
Sample	List of Pairs	Sample name from list of pairs
fwdReads	FASTQC	Number of forward reads contributing to the sample pair
fwdAvgLen	FASTQC	Average of all forward read lengths
fwdAvgQ	FASTQC	Average quality of all forward reads
revReads	FASTQC	Number of reverse reads contributing to the sample pair
revAvgLen	FASTQC	Average of all reverse read lengths
revAvgQ	FASTQC	Average quality of all reverse reads
percentHuman	Kraken2	Percentage of reads classified as *Homo sapiens*
readsHuman	Kraken2	Number of reads classified as *Homo sapiens*
percentSyntheticSeqs	Kraken2	Percentage of reads classified as non - biological sequences
readsSyntheticSeqs	Kraken2	Number of reads classified as non - biological sequences
percentCovid	Kraken2	Percentage of reads classified as SARS - CoV - 2
readsCovid	Kraken2	Number of reads classified as SARS - CoV - 2
avgReadCov	Bowtie2, samtools, ivar_trim	Average number of nts from sequence reads that map to the genome
percentReadsAlign	Bowtie2, samtools, Kraken2	Percentage of reads that aligned to the reference sequence
percentReadsPassFilt	Bowtie2, samtools, ivar_trim, Kraken2	Percentage of reads that pass the ivar_trim filter parameters: minReadLen = 30, minQual_slidingWindow = 20, and slidingWindow = 4 nt.
percent_nt0Xcov	Bowtie2, samtools, ivar_trim	Percentage of nucleotides that do not cover the genome at all (zero times)
percent_ntLess10Xcov	Bowtie2, samtools, ivar_trim	Percentage of nucleotides that barely cover the genome (less than 10 times)

6.3 **What is nucleotide coverage?! Let's look at 2 simple pictures**



In the figure above, let the burgundy line represent the entire reference genome.

The blue lines are the reads, as sequenced nucleotides.



In the figure above, each star, drawn on the burgundy line (reference genome) is a **nucleotide position**.

There are 28 stars, so we will say our genome is 28 nucleotides long.

We can use coverage to determine the quality of our sequences (blue lines).

The lime green stars along the genome represent 0X coverage, because we did not sequence any reads with **nucleotides positions covering that reference nucleotide position.** There are no blue lines that we sequenced there!

There are 3 nucleotide positions with 0x coverage. The total genome is 28 nucleotides long.

*percent_nt0Xcov = (nucleotidePositions0Xcov / genomeLength) * 100*

*percent_nt0Xcov = (3 / 28) *100*

percent_nt0xcov = 10.71%

In most ideal scenarios, higher coverage indicates better sequence quality.

For example, 100x coverage is better than 10x coverage.

Since we want **higher coverage**, percent_nt0Xcov and percent_ntLess10Xcov are ideally **lower percentages.**

0x coverage and 10x coverage indicate "no coverage" and "poor coverage", respectively.

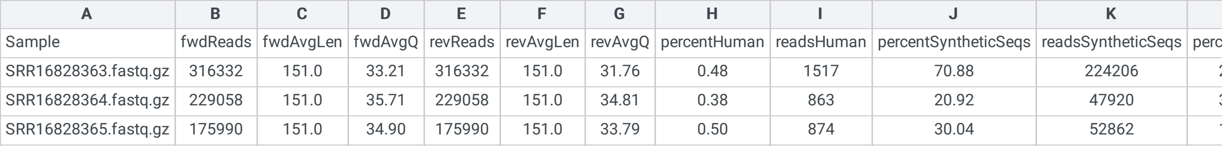
Generally, we expect avgReadCov in 100's or 1000's*****

If **percent_nt0Xcov** is a higher percentage, say 50%*****, that means half of the genome was not covered by our sequences. The quality is not ideal.

*** *These values are not official threshold and only used for illustrative purposes.*

Threshold guidance is 'in progress', and planned to be announced after further analyses.

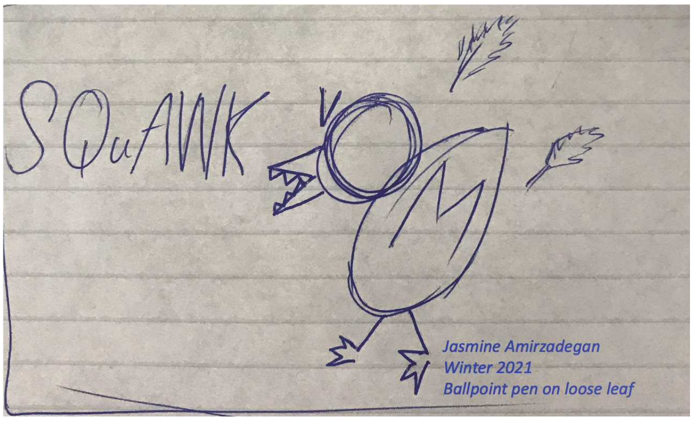
6.4 Example output for the first 3 pairs run through the SSQuAWK2 workflow:



A	B	C	D	E	F	G	H	I	J	K	
Sample	fwdReads	fwdAvgLen	fwdAvgQ	revReads	revAvgLen	revAvgQ	percentHuman	readsHuman	percentSyntheticSeqs	readsSyntheticSeqs	perc
SRR16828363.fastq.gz	316332	151.0	33.21	316332	151.0	31.76	0.48	1517	70.88	224206	2
SRR16828364.fastq.gz	229058	151.0	35.71	229058	151.0	34.81	0.38	863	20.92	47920	3
SRR16828365.fastq.gz	175990	151.0	34.90	175990	151.0	33.79	0.50	874	30.04	52862	1

Video Tutorial

7 Thanks for using SSQuAWK2!



8 **New to GalaxyTrakr? Check out this detailed, 12 minute video overview of the SSQuAWK (version 1)**

protocol before trying SSQuAWK2.

Video edit:
"SSQuawk allows users to check the sequence quality of SARS-CoV-2 wastewater samples in ***CFSAN's custom Galaxy instance, called GalaxyTrakr.*** *This generates a single report file from raw Illumina MiSeq paired-end fastq file inputs."*

Citation: Jasmine Amirzadegan, Tunc Kayikcioglu, hugh.rand , Ruth Timme, Maria Balkey Wastewater QC workflow in GalaxyTrakr (SSQuAWK2) https://dx.doi.org/10.17504/protocols.io.b5u2q6ye