Unlocking the Microbial Mysteries in Children with Special Needs: A High School Journey Through the Gut

Grades 9 - 12 Edition

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Background and Introduction.

Autism spectrum disorder is a developmental disability caused by differences in the brain. This causes people to have restricted behavior, problems with communication/paying attention or even physical learning challenges. There are a wide range of conditions that affect 1 in 44 children today. Autism symptoms, especially with more advanced cases, appear around the ages of 12-14 months or earlier.

Objectives.

- Identifying taxonomic differences between healthy patients and individuals with autism spectrum disorder using publicly available datasets and bioinformatics tools
- Understanding the implications of gut microbes in human health
- Developing education modules that can be disseminated into schools to promote STEM.

Student Background Knowledge.

Part I. Domains of life. The three domains of life are Archaea, Bacteria and Eukarya. Members of the domain Bacteria are unicellular and prokaryotic. This domain corresponds to the kingdom Eubacteria. Microbiome is a collection of all microbes, both commensal and pathogenic, that live in and on human bodies. A very well studied microbiome is the gut microbiome. Other examples are skin, oral, uro-genital, etc.

Part II. Gut microbiota- definition, function and autism.

These are the type of microbiota that live in the digestive tracts of vertebrates and invertebrates. It refers to microbes and bacterial species that live in your body- they help with digestion, destroy harmful bacteria and helps control the immune system. Microbes can be both helpful and potentially harmful. Most of them have symbiotic relationships with the human body where they both benefit while a few are pathogenic which spread/carry disease.

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Benefits to having microbiota:

- Stimulate the immune system, meaning they aid in the breakdown of food compounds and synthesize certain vitamins and amino acids.
- Smaller compounds like table sugar and lactose can be absorbed quickly in the small intestine, microbiota help break down larger particles such as complex carbohydrates and starch with their digestive enzymes.

An imbalance of unhealthy and healthy gut microbiota can contribute to weight gain, high blood sugar, high cholesterol, and neuro-developmental conditions.

Many studies suggest gut microbes influence many neurological disorders, for example, autism. It has been found that patients with autism do not have a healthy gut. There is mounting evidence suggesting pathways between gut bacteria and the central nervous system, referred to as the gut—brain axis, which a profound effect on social behaviors.

Part III. DNA Sequencing.

Sequencing DNA means determining the order of the four chemical building blocks - called "bases" - that make up the DNA molecule. The sequence tells scientists the kind of genetic information that is carried in a particular DNA segment. For example, scientists can use sequence information to determine which stretches of DNA contain genes and which stretches carry regulatory instructions, turning genes on or off. In addition, and importantly, sequence data can highlight changes in a gene that may cause disease.

In the DNA double helix, the four chemical bases always bond with the same partner to form "base pairs." Adenine (A) always pairs with thymine (T); cytosine (C) always pairs with guanine (G). This pairing is the basis for the mechanism by which DNA molecules are copied when cells divide, and the pairing also underlies the methods by which most DNA sequencing experiments are done. The human genome contains about 3 billion base pairs that spell out the instructions for making and maintaining a human being.

Part IV. 16S rRNA Sequencing.

16S rRNA gene is used as a marker for identification and taxonomic classification of microbes. Since this gene is ubiquitous and highly conserved among bacteria as well as archaea, it is popularly used for phylogenetic studies.

The 16S rRNA gene consists of both conserved and variable regions. While the conserved region makes amplification possible through PCR, sequencing the variable regions allows distinction between different microorganisms such as bacteria and archaea.

Conserved- present in everything - not studied.

Hypervariable region- part that is studied.

Primers - enable you to read through hypervariable regions- present in both directions.

Part VI. Gut Microbiome and Autism Spectrum Disorder.

Research into the relationship between the gut microbiome and autism spectrum disorder (ASD) is an emerging and evolving field. While there is no conclusive evidence that links gut microbiota directly to the development of ASD, there is growing interest in understanding the potential connections between the two. Here are some key points and findings regarding the gut microbiome and ASD:

- 1. Gut-Brain Axis: The gut-brain axis is a bi-directional communication system between the gut and the brain. Emerging research suggests that the composition of the gut microbiota may influence brain function and behavior. Alterations in the gut microbiome could potentially impact neurological and behavioral development, which is relevant to ASD.
- 2. Microbiome Composition: Some studies have found differences in the gut microbiota of individuals with ASD compared to neurotypical individuals. These differences often involve changes in the relative abundance of certain bacterial species. However, these findings are not consistent across all studies, and there is a need for more research to establish clear patterns.
- 3. Potential Mechanisms: Several potential mechanisms have been proposed to explain how gut microbiota might influence ASD. These include the production of metabolites (such as shortchain fatty acids) that can affect the central nervous system, the modulation of the immune system, and the influence on the production of neurotransmitters.
- 4. Immune System: The gut microbiome plays a crucial role in the development and maintenance of the immune system. Dysregulation of the immune system has been observed in some individuals with ASD, and there is evidence to suggest that the gut microbiota can influence immune function.
- 5. Clinical Implications: While the link between gut microbiota and ASD is still not fully understood, some researchers and clinicians have explored potential dietary and probiotic interventions to modulate the gut microbiome as a complementary approach to managing certain symptoms of ASD. However, the effectiveness of these interventions remains a subject of ongoing investigation.
- 6. Limitations: It's essential to recognize that the research on the gut microbiome and ASD is in its early stages. Many studies are small and may have methodological limitations, making it challenging to draw definitive conclusions. In summary, there is growing interest in the potential role of the gut microbiome in ASD. While some research suggests there may be associations between gut microbiota composition and ASD, more extensive and rigorous studies are needed to establish the nature and mechanisms of this relationship.

Vocabulary

Microbiome: The collective term for all microorganisms, including bacteria, viruses, fungi, and archaea, that inhabit a particular environment.

Gut Microbiome: The gut microbiome refers to the community of microorganisms, including bacteria, viruses, fungi, and other microorganisms, that inhabit the human gastrointestinal tract.

ASD: Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition that affects an individual's social interaction, communication, behavior, and sensory processing. It is called a "spectrum disorder" because it encompasses a wide range of symptoms and severity levels, meaning that no two individuals with ASD are exactly alike. The term "spectrum" acknowledges that the condition can manifest differently in each person.

16S rRNA sequencing: A common technique used to analyze the microbial composition in a sample by targeting and sequencing a specific region of the 16S ribosomal RNA gene, which is highly conserved in bacteria and archaea. It helps identify and classify bacterial species.

Microbiome profiling: The process of characterizing and quantifying the composition of the microbiome in each sample.

Taxonomy: The classification of microorganisms into hierarchical categories, such as phylum, class, order, family, genus, and species.

Bioinformatics: Bioinformatics is an interdisciplinary field that merges computers and biology in order to analyze large datasets (such as DNA sequences) to discover new, biologically important knowledge.

Materials Checklist

Access to a laptop or desktop computer. Access to datasets Galaxy online server

Classroom Management

Overview.

Students are given the pdf document and required to read. Understand what gut microbiome is in the context of autism and why studying differences in microbial composition is important.

Pre-class preparation.

Ensure there is internet access for the activity along with limited storage on computer. If computers are limited, students can work in groups.

In-class activity

Go through activity step-by-step. Look at data and answer questions that follow post bioinformatics analysis.

References

Chiappori, Federica, Francesca Anna Cupaioli, Arianna Consiglio, Noemi Di Nanni, Ettore Mosca, Vito Flavio Licciulli, and Alessandra Mezzelani. "Analysis of Faecal Microbiota and Small ncRNAs in Autism: Detection of miRNAs and piRNAs with Possible Implications in Host–Gut Microbiota Cross-Talk." Nutrients 14, no. 7 (2022): 1340.

Xu, YuShuang, YiHua Wang, JinShuang Xu, Yu Song, BingQiang Liu, and ZhiFan Xiong. "Leveraging existing 16SrRNA microbial data to define a composite biomarker for autism spectrum disorder." Microbiology Spectrum 10, no. 4 (2022): e00331-22.

Questions

1)	What do the different colors in the bar plots mean?	
2)	What are the differences (microbial composition) between healthy patients and compromised patient samples?	
3)	Describe what is on the x and y axis?	
4)	In addition to autism cvan you list other factor that influence gut microbiome?	

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Tutorial Instructions created by Trisha Raichura, Dubai College, Dubai, UAE.

We will be using https://cancer.usegalaxy.org/ online web server to perform our analysis.

We will be using the following dataset.

CONTROL	AUTISM SPECTRUM DISORDER (ASD)
SRR18253050_ctrl01_16_16S_07CSV16.extendedFrags.subsample.fastq.gz	SRR18253070_asd01_16_16S_01ASV16.extendedFrags.subsample.fastq.gz
SRR18253049_ctrl02_16_16S_08CSV16.extendedFrags.subsample.fastq.gz	SRR18253069_asd02_16_16S_02ASV16.extendedFrags.subsample.fastq.gz
SRR18253048_ctrl03_16_16S_10CSV16.extendedFrags.subsample.fastq.gz	SRR18253058_asd03_16_16S_03ASV16.extendedFrags.subsample.fastq.gz
SRR18253047_ctrl04_16_16S_11CSV16.extendedFrags.subsample.fastq.gz	SRR18253053_asd04_16_16S_04ASV16.extendedFrags.subsample.fastq.gz
SRR18253068_ctrl05_16_16S_12CSV16.extendedFrags.subsample.fastq.gz	SRR18253052_asd05_16_16S_05ASV16.extendedFrags.subsample.fastq.gz
SRR18253067_ctrl06_16_16S_13CSV16.extendedFrags.subsample.fastq.gz	SRR18253051_asd06_16_16S_06ASV16.extendedFrags.subsampled.fastq.gz

The files are approximately 2 MB in size. The data was part of the project published in the following study, Xu, Y., Wang, Y., Xu, J., Song, Y., Liu, B., & Xiong, Z. (2022). Leveraging existing 16SrRNA microbial data to define a composite biomarker for autism spectrum disorder. Microbiology Spectrum, 10(4), e00331-22. The authors made the data publicly in NCBI under Bioproject ID PRJNA813424.

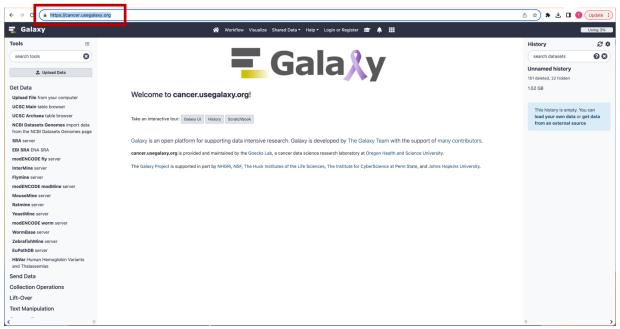
Data will be made available through google folder for students and teachers for downloading it onto their local machine before starting the analysis.

QIIME2 (https://qiime2.org/) will be used for exploring the bacterial diversity in the control versus ASD samples. QIIME2 is a collection several commands and we will using a subset of these commands, listed below for analysis.

QIIME2 Commands:

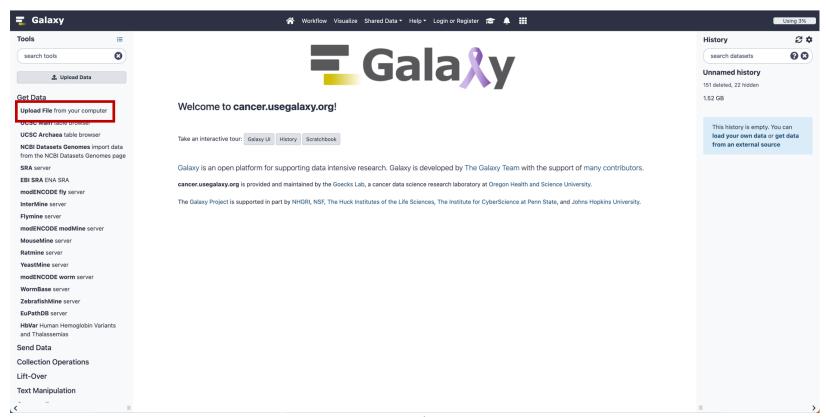
```
qiime2 tools import
qiime2 dada2 denoise-single
qiime2 feature-classifier classify-sklearn
qiime2 metadata tabulate
qiime2 feature-table tabulate-seqs
qiime2 feature-table filter-samples
qiime2 taxa bar-plot
```

STEP 1: Use a browser preferably latest version of google chrome to access https://cancer.usegalaxy.org/



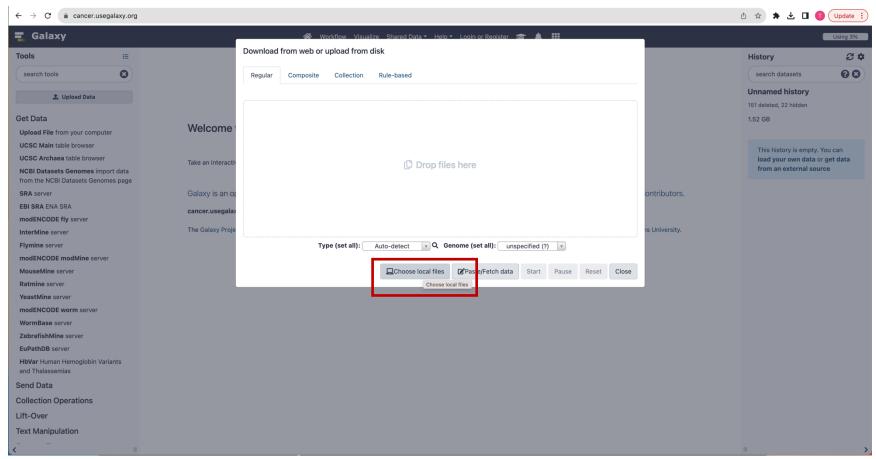
Screenshot 1

STEP 2: Use "Upload File from your computer" option to load compressed FASTQ files of all the samples.



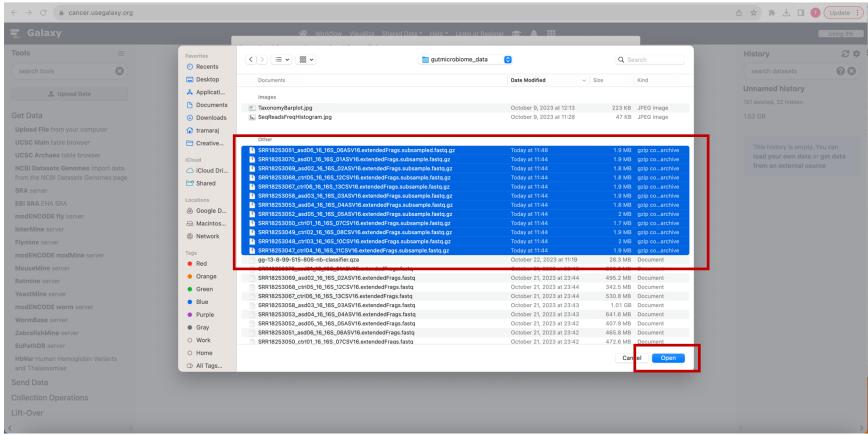
Screenshot 2

STEP3: Click "Choose local files" option to upload files from your computer.



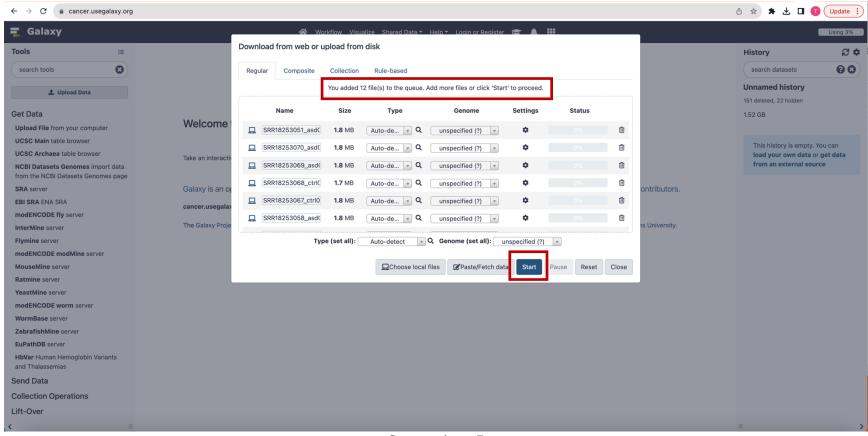
Screenshot 3

<u>STEP 4:</u> Go to appropriate folder and load required files for analysis by selecting all the files and clicking "Open".



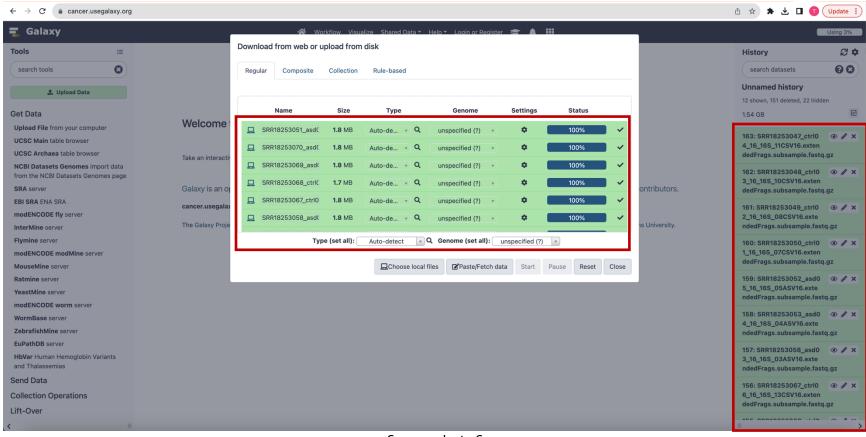
Screenshot 4

STEP 5: You should be able to see all the added files. Click "Start" to proceed with uploading the files onto the server.



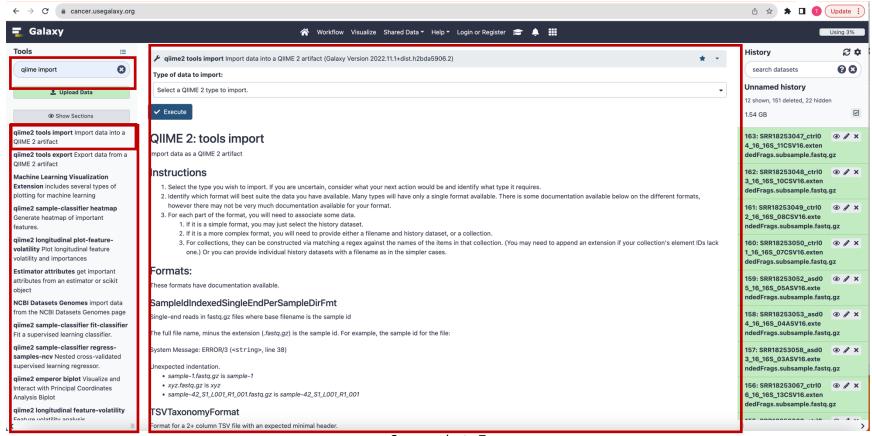
Screenshot 5

<u>STEP 6:</u> We should see the status to be 100% for all the files and the files should be visible in the history (left hand side)



Screenshot 6

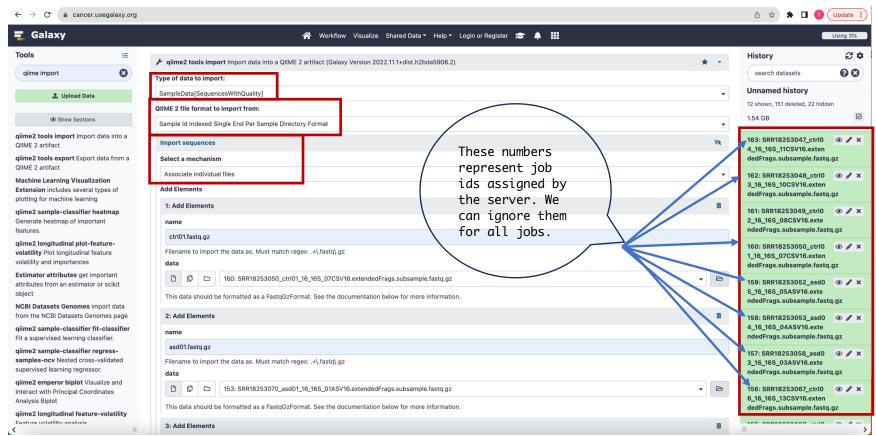
STEP 7: At this point we have all the files ready to be analyzed. Starting from this step we will be executing QIIME2 steps to investigate the gut microbiome community. To run QIIME2 the uploaded compressed FASTQ files should be imported as a QIIME2 artifact. To accomplish this, we will run the "qiime tools import" command. Using the search bar under "Tools" on the left-hand side type "qiime import" and hit "return" to complete search. You should be able to see the search results displayed on the left-hand side as well. From the search results select "qiime2 tools import Import data into a QIIME 2 artifact"



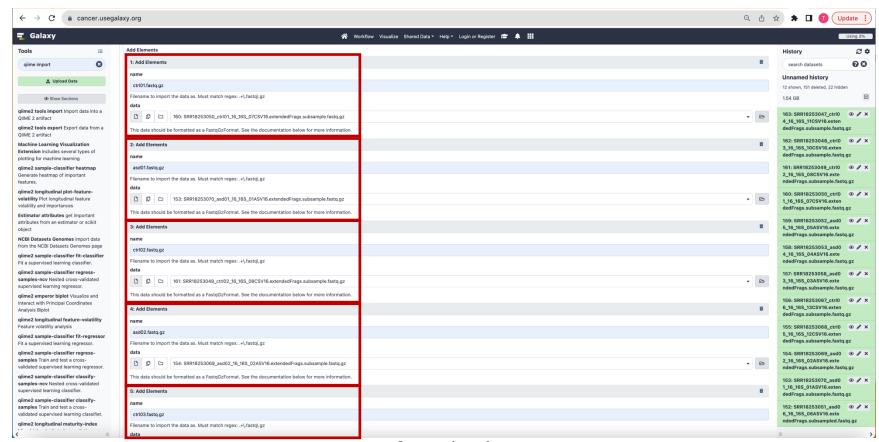
Screenshot 7

STEP 8: Here we will input required parameters for "qiime2 tools import".

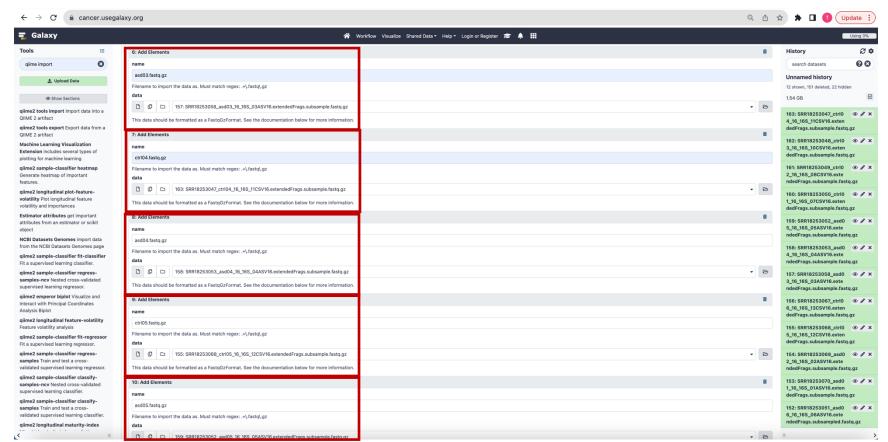
- FOR "Type of data to import:" SELECT "SampleData[SequencesWithQuality]
- FOR "QIIME 2 file format to import from:" SELECT "Sample Id Indexed Single End Per Sample Directory Format".
- UNDER "Import sequences" FOR "Select a mechanism" SELECT "Associate Individual Files".
- UNDER "Import sequences" FOR "Add Elements" you will add 12 elements for this analysis, 6 control (ctrl) samples and 6 autism spectrum disorder (asd) samples.
- For each element you will be providing a name for instance first element FOR "name" is assigned "ctrl01.fastq.gz" and SELECT "SRR18253050_ctrl01_16_16S_07CSV16.extendedFrags.subsample.fastq.gz" from the list.
- Screenshots 8 11 illustrates how to input all the parameters and hit "Execute".
- Screenshot 12 "qiime2 tools import" job has been submitted and is being executed. We can see that 12 files are being used as input and we will get file as output representing qiime2 artifact file which will be used for further downstream analysis.



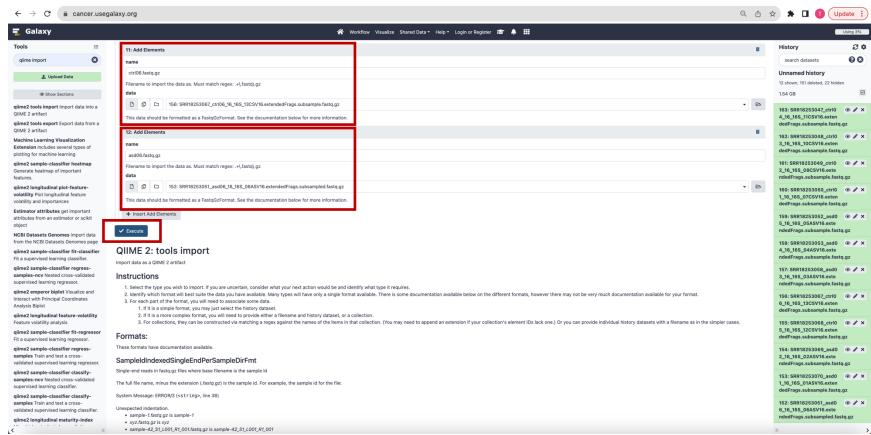
Screenshot 8



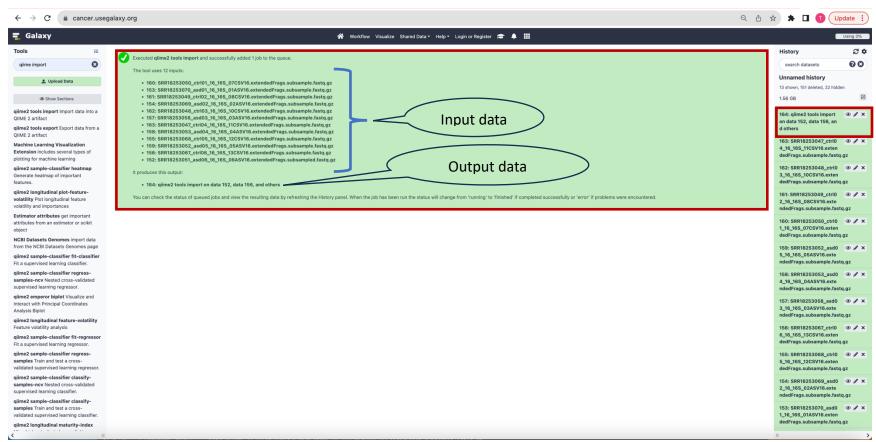
Screenshot 9



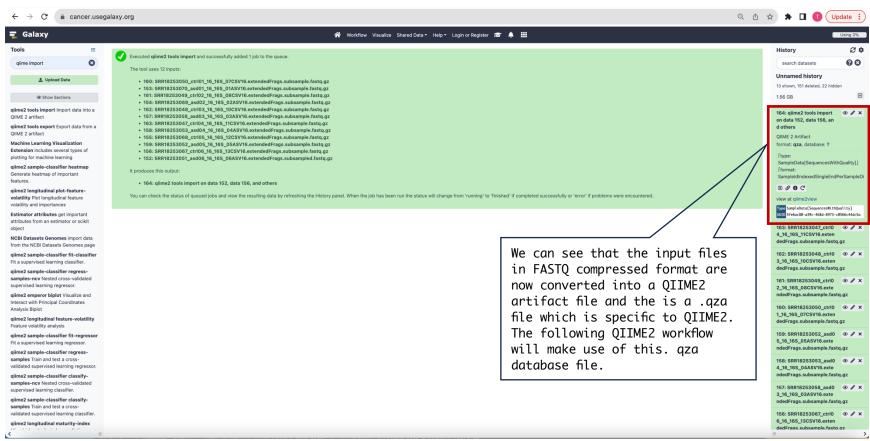
Screenshot 10



Screenshot 11



Screenshot 12

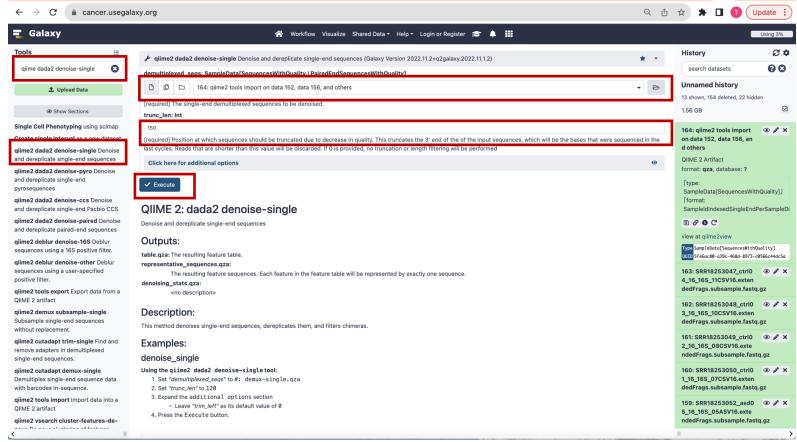


Screenshot 13

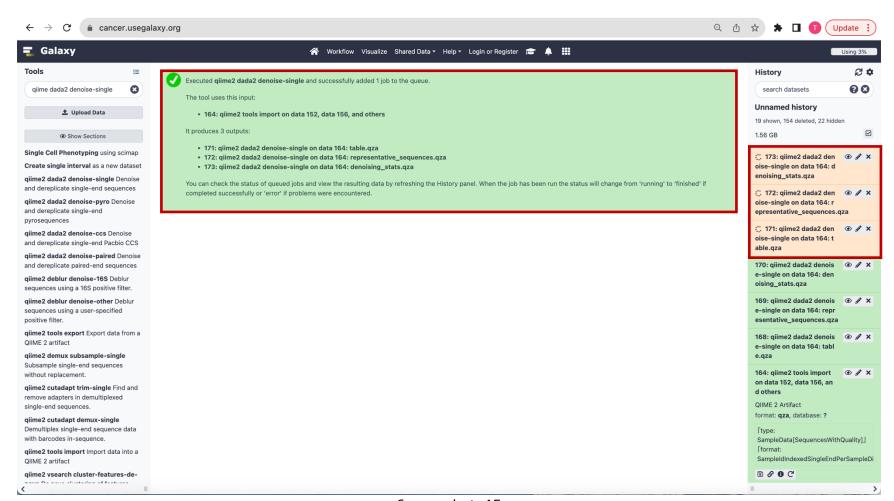
STEP 9: Running "qiime2 dada2 denoise-single".

FOR "demultiplexed_seqs:SampleData[SequencesWithQuality[PairedEndSequencesWithQuality] SELECT results from previous step as show in the screenshot below

For trunc len: Int ENTER 150 and HIT "Execute".



Screenshot 14



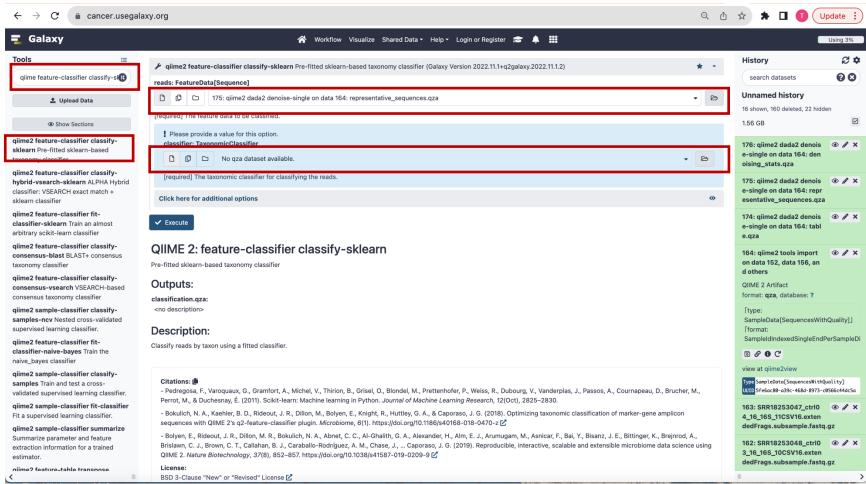
Screenshot 15

STEP 10: Run "qiime2 feature-classifier classify-sklearn"

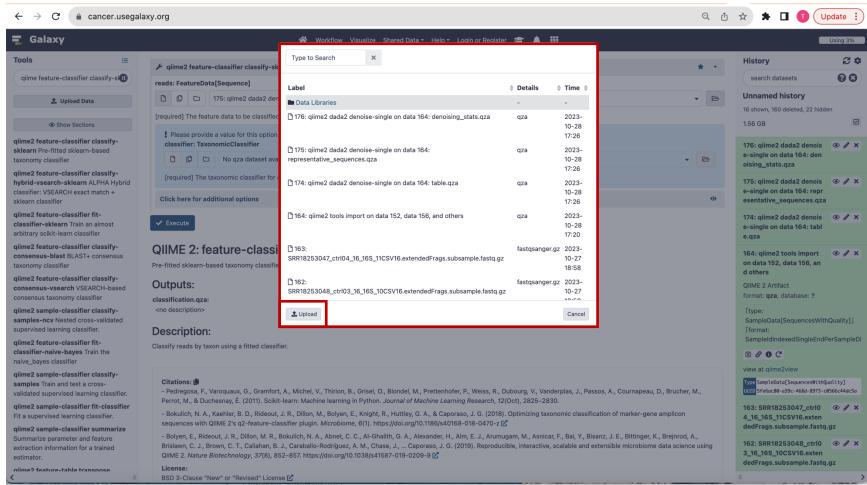
FOR reads: FeatureData[Sequence] SELECT option highlighted in the box.

FOR classifier:taxonomicCLassifier follow instructions shown in screenshots 17 - 21

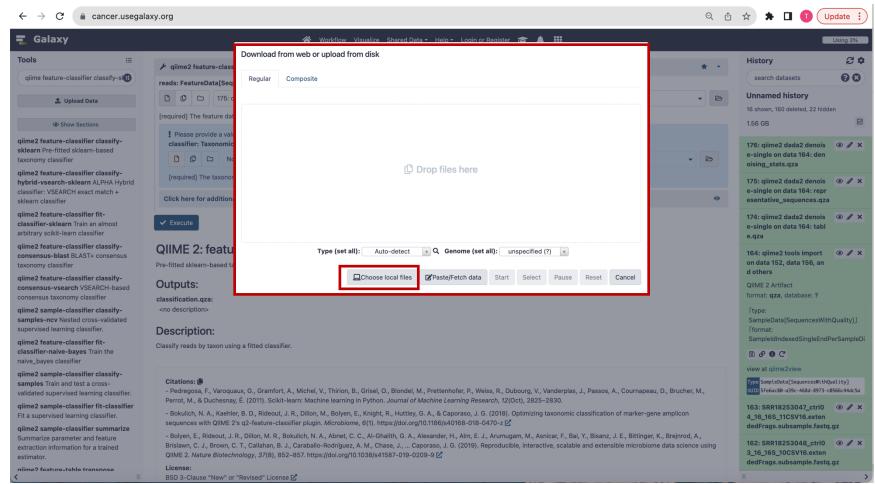
Screenshot 22: All required input values have been entered, hit "Execute"



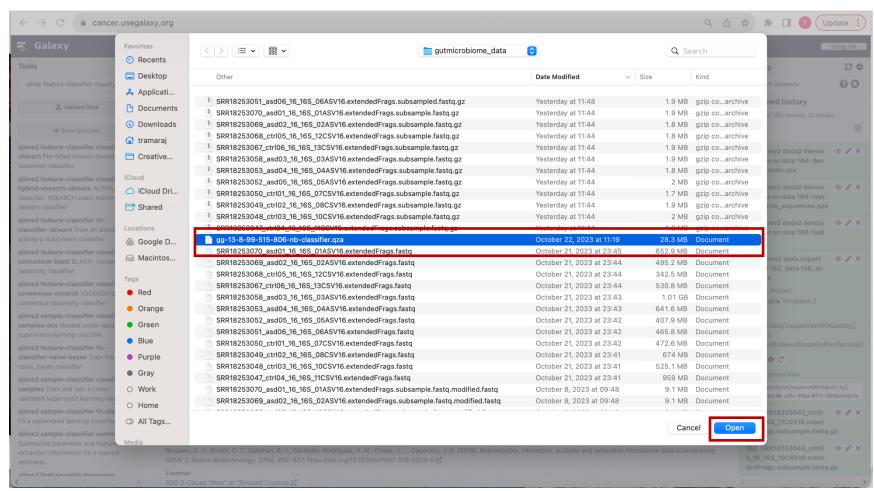
Screenshot 16



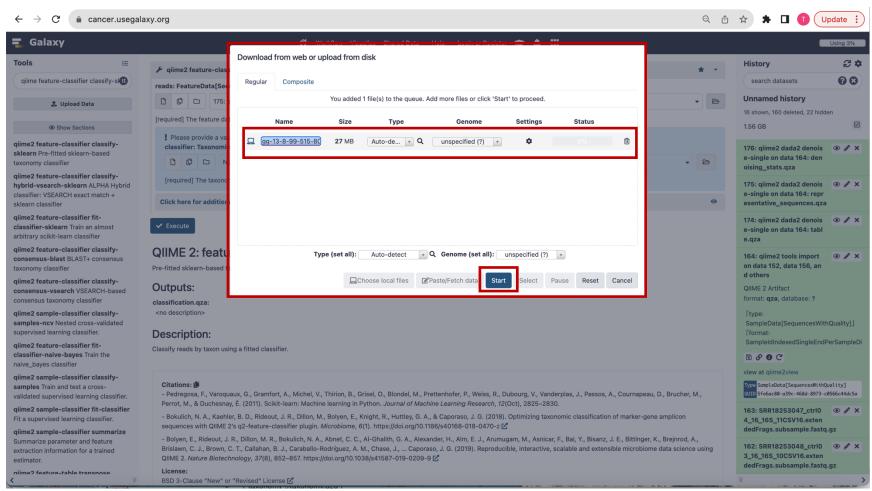
Screenshot 17



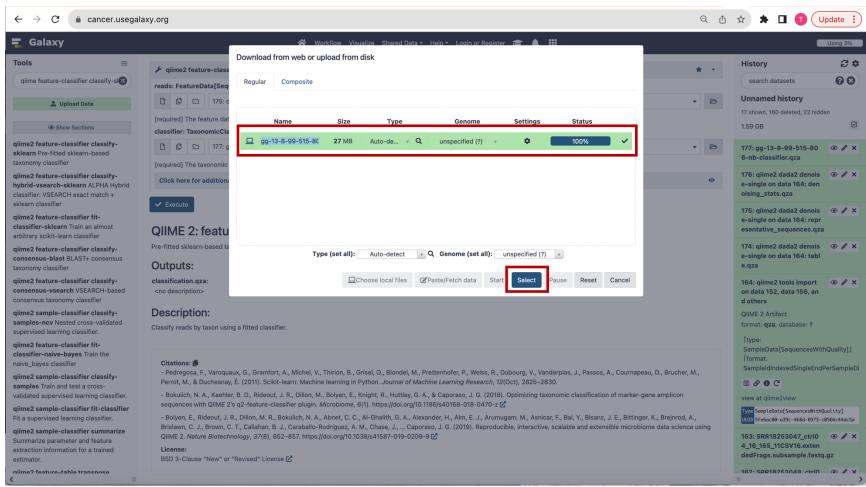
Screenshot 18



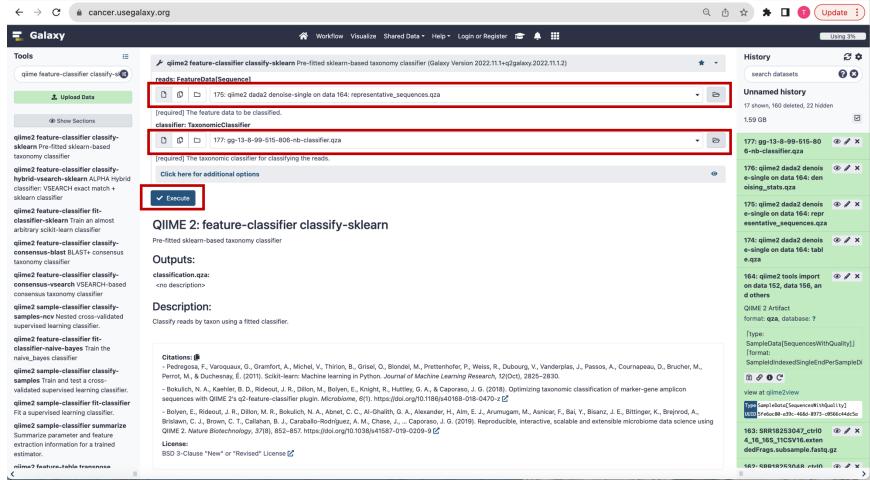
Screenshot 19



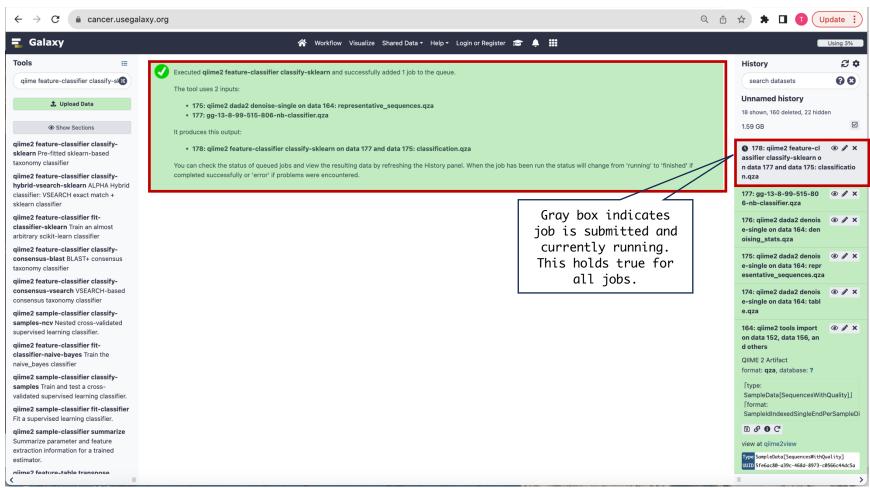
Screenshot 20



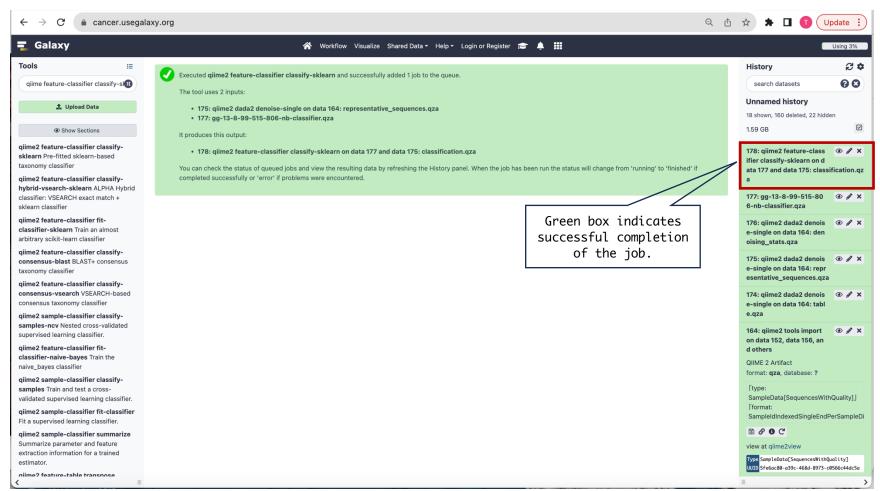
Screenshot 21



Screenshot 22



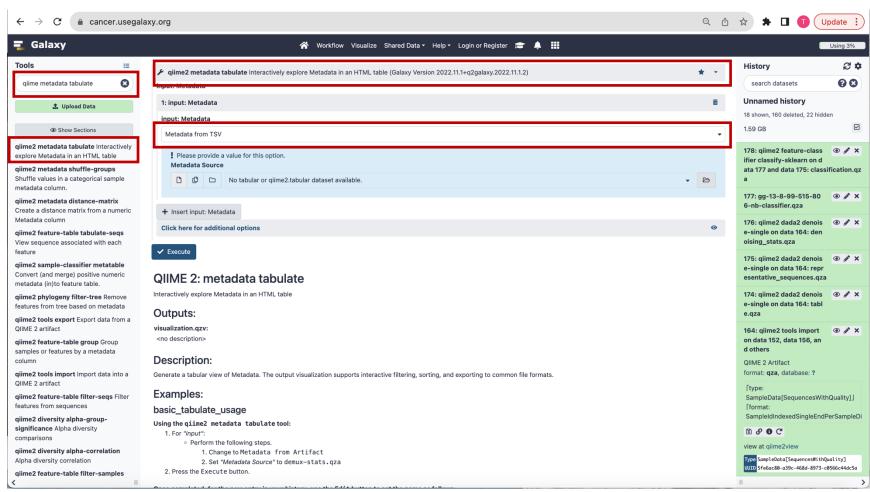
Screenshot 23



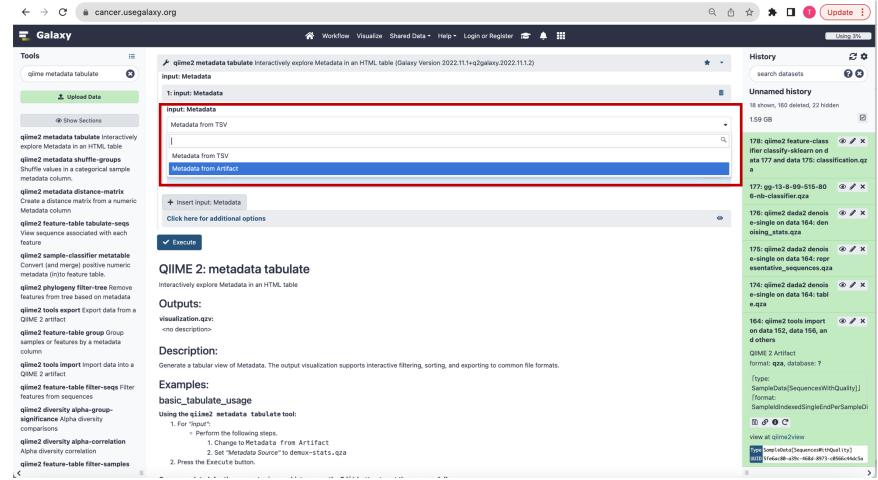
Screenshot 24

STEP 11: Run "qiime2 metadata tabulate".

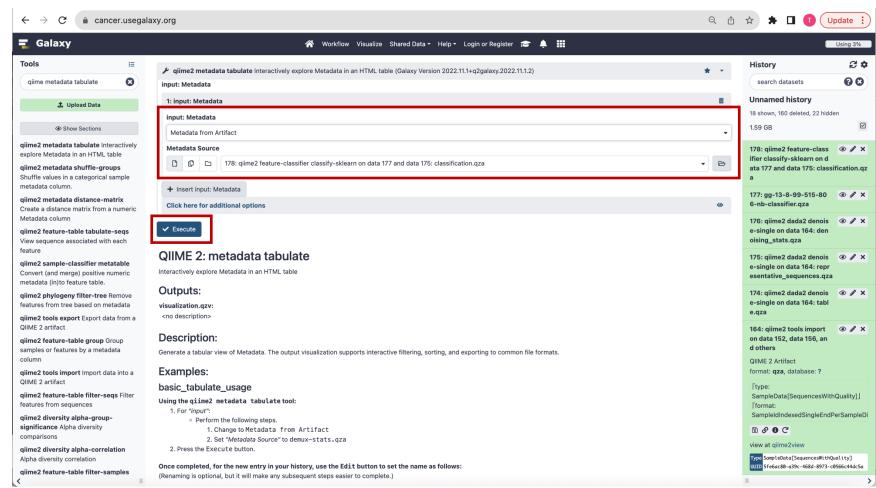
FOR input:Metadata SELECT "Metadata from Artifact" AND "Execute" as shown in screenshot 25 - 28.



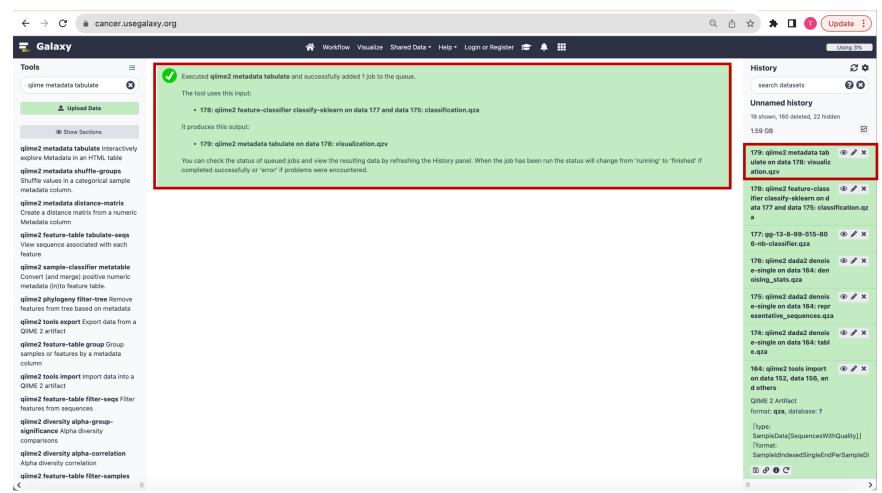
Screenshot 25



Screenshot 26



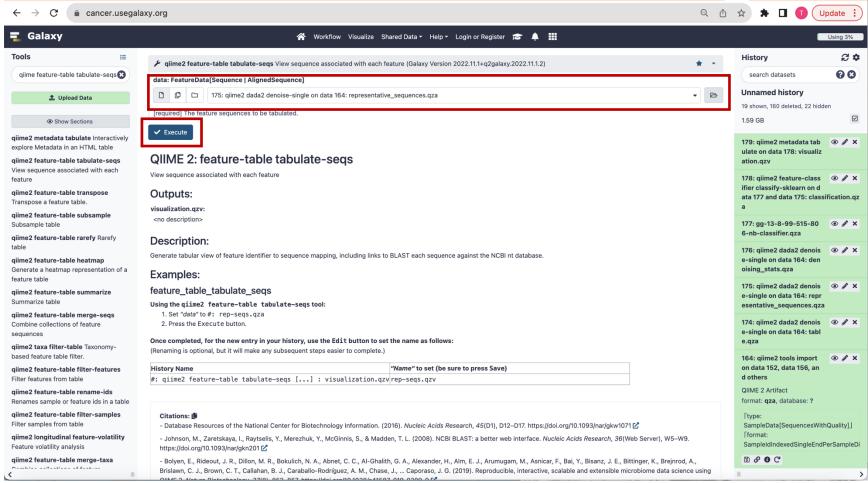
Screenshot 27



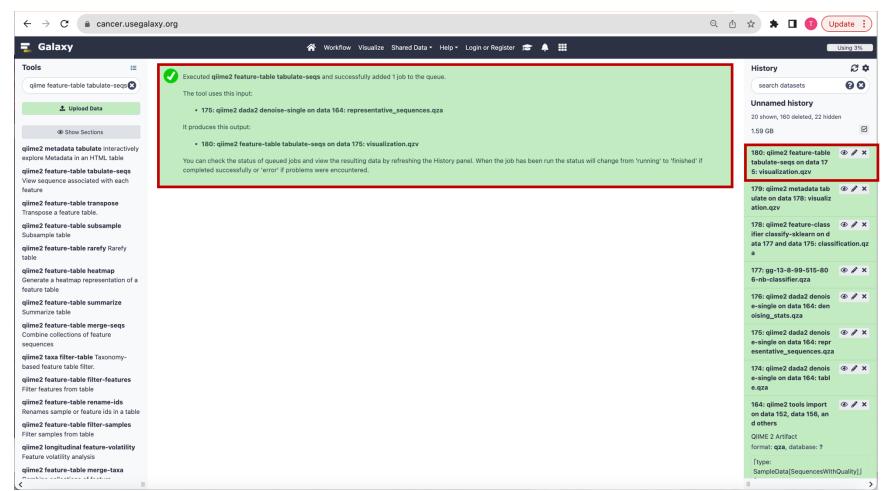
Screenshot 28

STEP 12: Run "qiime2 feature-table tabulate-seqs".

FOR data:FeatureData[Sequence|AlignedSequence] SELECT option highlighted in the box AND "Execute" as illustrated in screenshot 29 - 30.



Screenshot 29

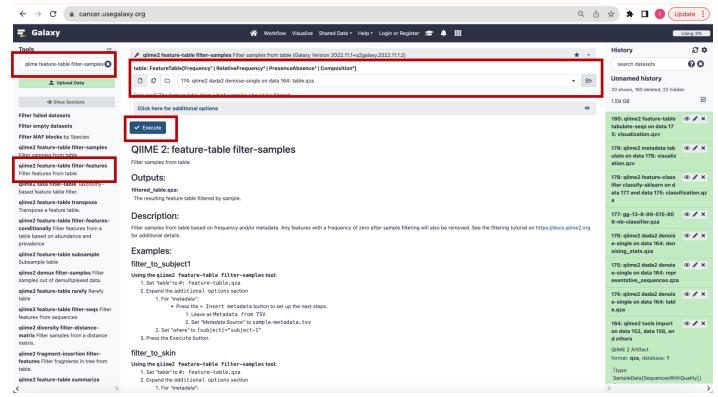


Screenshot 30

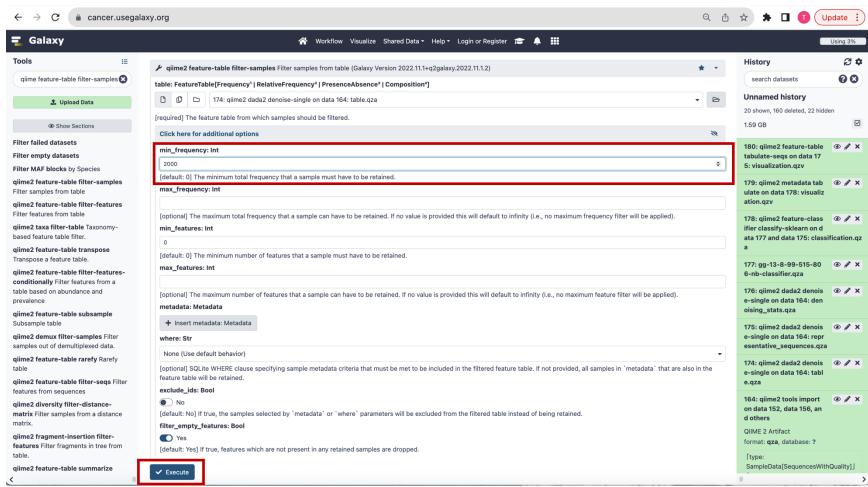
STEP 13: Run "qiime feature-table filter-samples".

FOR table:FeatureTable[Frequency¹|RelativeFrequency²|PresenceAbsence³|Composition⁴] SELECT option highlighted in the box.

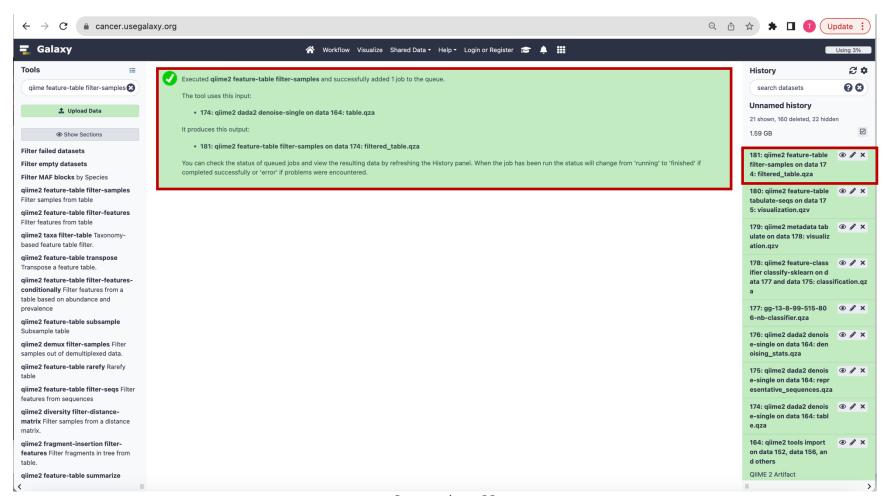
For this command we must input an additional parameter listed under "Click here for additional options". Follow screenshots 31 – 33 to fulfill this requirement.



Screenshot 31



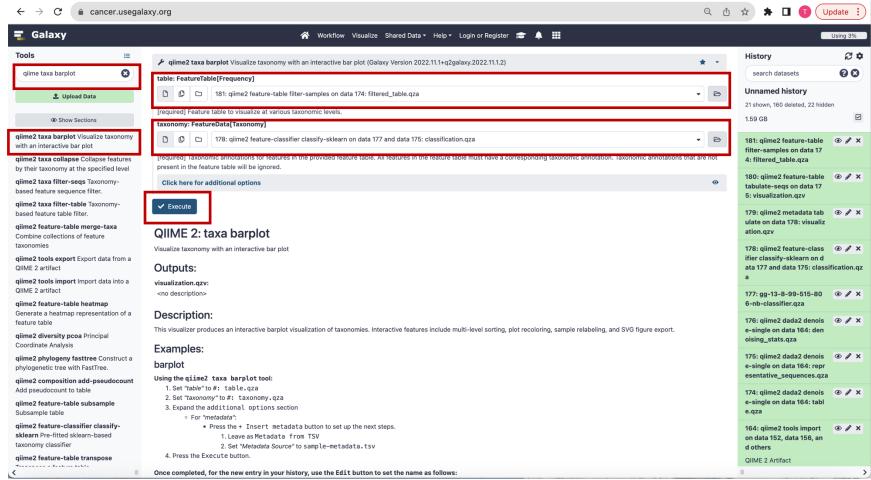
Screenshot 32



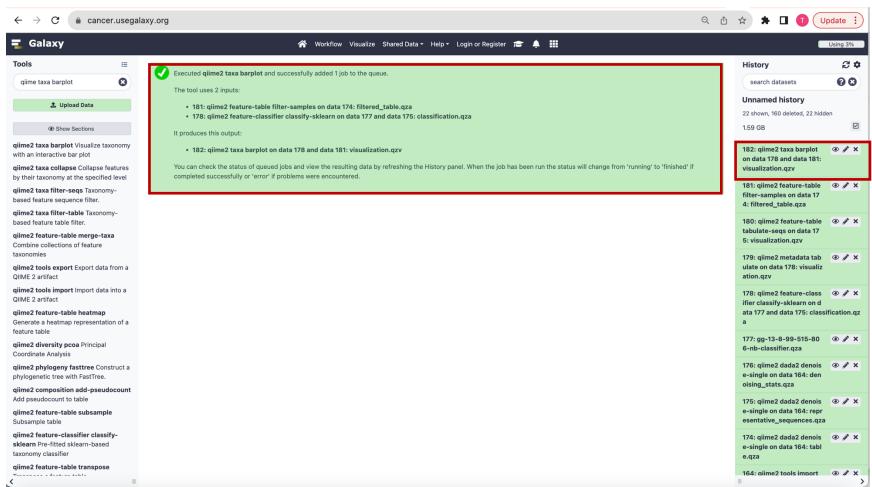
Screenshot 33

STEP 13: Run "qiime2 taxa barplot".

FOR table:FeatureTable[Frequency] AND taxonomy:FeatureData[Taxonomy] SELECT options highlighted in the box AND "Execute" as shown in screenshot 34 - 36.

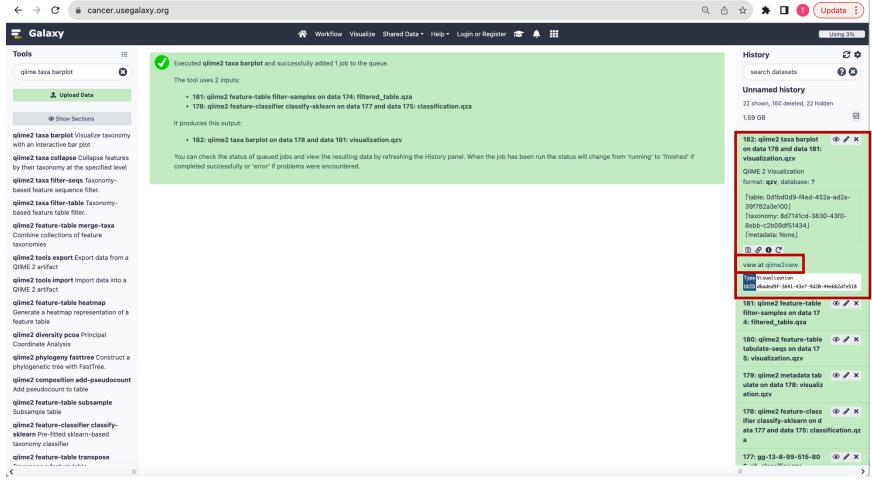


Screenshot 34

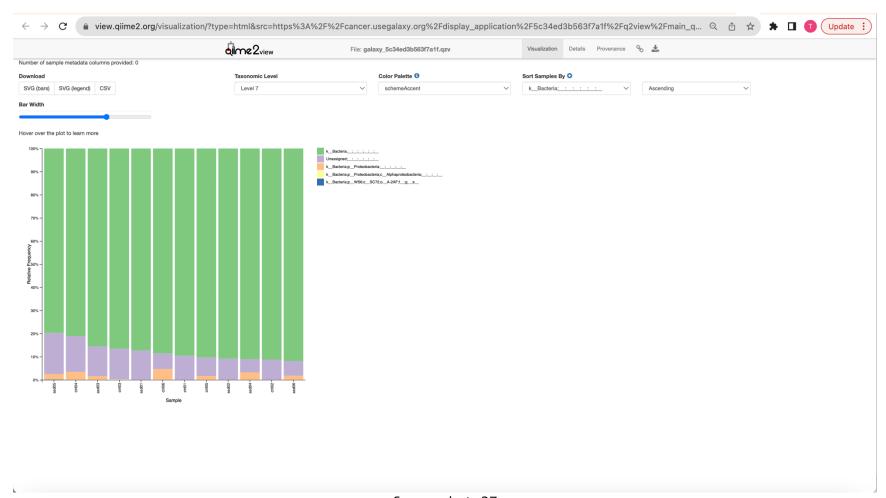


Screenshot 35

To visualize the taxonomies, click "view at qiime2view" option listed in the right-hand box. This will open a new page in the browser. It will be an interactive page which will let user interactively explore the taxonomies in different samples. An example screenshot of the webpage is shown in screenshot 37.



Screenshot 36



Screenshot 37

Preliminary results from this analysis represents similar results described in the published article.

Reference: Chiappori, F., Cupaioli, F.A., Consiglio, A., Di Nanni, N., Mosca, E., Licciulli, V.F. and Mezzelani, A., 2022. Analysis of Faecal Microbiota and Small ncRNAs in Autism: Detection of miRNAs and piRNAs with Possible Implications in Host-Gut Microbiota Cross-Talk. Nutrients, 14(7), p.1340.