

Analyzing Variant Call results using EuPathDB Galaxy, Part II

In this exercise we will work in groups to examine the results from the SNP analysis workflow that we started yesterday. *The first step is to share your SNP workflow histories with the rest of the workshop participants:*

1. Give your workflow a meaningful name, eg. The sample or group name.
2. Click on the on the 'History options' link and select the 'share or Publish option'.
3. On the next page click on the 'Make History Accessible and Publish' link.

1

History

search datasets

ENU-mutant RH clone resistant to IBET-151 1C6
7 shown, 12 hidden

52.95 GB

17: SnpEff on data 15

16: SnpEff on data 15

13: BAM to BigWig on data 12

9: FastQC on data 2: Webpage

3: FastQC on data 1: Webpage

2: SRR5123637_2.fastq.gz

1: SRR5123637_1.fastq.gz

2

History

HISTORY LISTS

search

Saved Histories

Histories Shared with Me

CURRENT HISTORY

Create New

Copy History

Copy Datasets

Share or Publish

Extract Workflow

Dataset Security

Resume Paused Jobs

Collapse Expanded Datasets

Unhide Hidden Datasets

Delete Hidden Datasets

Purge Deleted Datasets

Show Structure

Export Citations

Export to File

Delete

Delete Permanently

OTHER ACTIONS

3

Share or Publish History 'ENU-mutant RH clone resistant to IBET-151 1C6'

Make History Accessible via Link and Publish It

This history is currently restricted so that only you and the users listed below can access it. You can:

Make History Accessible via Link

Generates a web link that you can share with other people so that they can view and import the history.

Make History Accessible and Publish

Makes the history accessible via link (see above) and publishes the history to Galaxy's Published Histories section, where it is publicly listed and searchable.

Share History with Individual Users

You have not shared this history with any users.

Share with a user

[Back to Histories List](#)

History

search datasets

ENU-mutant RH clone resistant to IBET-151 1C6
7 shown, 12 hidden

52.95 GB

17: SnpEff on data 15

16: SnpEff on data 15

13: BAM to BigWig on data 12

9: FastQC on data 2: Webpage

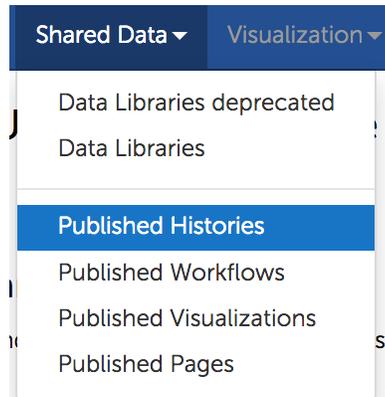
3: FastQC on data 1: Webpage

2: SRR5123637_2.fastq.gz

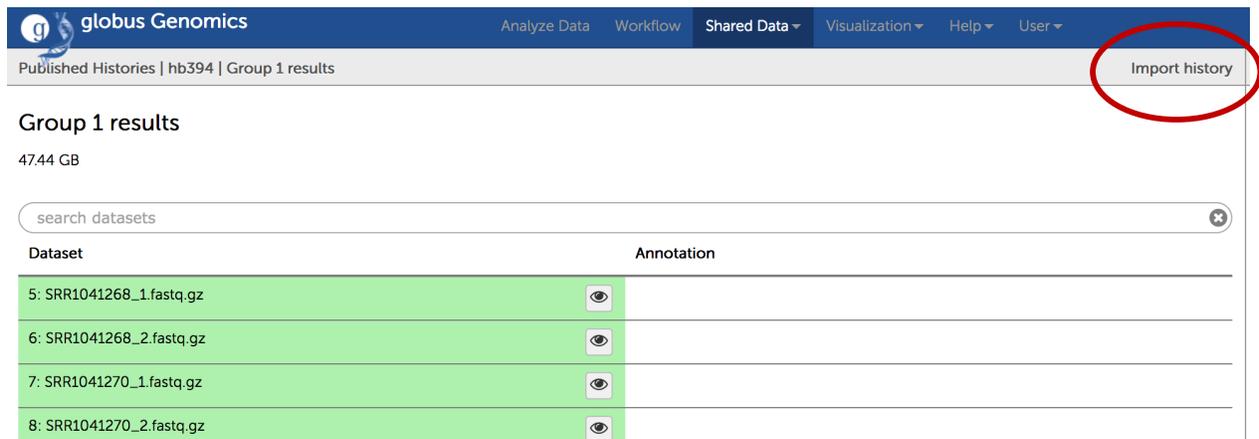
1: SRR5123637_1.fastq.gz

To import a shared history into your workspace follow these steps:

1. Select 'Published Histories' from the Shared data menu.



2. From the list of shared histories click on the one you want to import and on the next page select the 'Import' link in the upper right hand side.

A screenshot of the 'Group 1 results' page in the globus Genomics interface. The page header includes the globus Genomics logo and navigation links for 'Analyze Data', 'Workflow', 'Shared Data', 'Visualization', 'Help', and 'User'. Below the header, the page title is 'Published Histories | hb394 | Group 1 results'. In the top right corner, there is a button labeled 'Import history' which is circled in red. Below the header, the page displays 'Group 1 results' with a size of '47.44 GB'. There is a search bar labeled 'search datasets'. Below the search bar is a table with two columns: 'Dataset' and 'Annotation'. The table contains four rows of data, each with a green background and an eye icon in the 'Annotation' column.

Dataset	Annotation
5: SRR1041268_1.fastq.gz	
6: SRR1041268_2.fastq.gz	
7: SRR1041270_1.fastq.gz	
8: SRR1041270_2.fastq.gz	

Examining your results:

1. Click on the hidden files link in the history panel to reveal all workflow output files.

The image displays two side-by-side screenshots of a workflow history panel. The left screenshot shows a workflow named "B. micro Wisconsin single" with 4 shown and 7 hidden files. A red circle highlights the "7 hidden" text, and a red arrow points to the right screenshot. The right screenshot shows the same workflow with 11 files shown, including several hidden datasets indicated by orange warning boxes with "Unhide it" links.

Workflow Step	Visibility
B. micro Wisconsin single	4 shown, 7 hidden
11: SnpEff on data 9	Visible
10: SnpEff on data 9	Visible
3: FastQC on data 1: RawData	Visible
1: ERR1349056.fastq.gz	Visible
9: Filter variants by quality on data 8: filtered by quality	Hidden
8: FreeBayes on data 7 (variants)	Hidden
7: Sort on data 6: sorted BAM	Hidden
6: Bowtie2 on data 4: aligned reads	Hidden

2. Examine the output files. What does the tool FASTQC do? What about Sickle?
3. The output of Sickle is used by a program called Bowtie2. What does this tool do? Bowtie generates a file called a BAM file. Whenever dealing with sequence alignment files you will likely hear of file formats called SAM or BAM. SAM

stands for Sequence Alignment/Map format, and BAM is the binary version of a SAM file.

4. Many of the downstream analysis programs that use BAM files require a sorted BAM file. This allows access to reads to be done more efficiently.
5. The sorted BAM file is the input for a program called FreeBayes. This program is a Bayesian genetic variant detector designed to find small polymorphisms, specifically SNPs (single-nucleotide polymorphisms), indels (insertions and deletions), MNPs (multi-nucleotide polymorphisms), and complex events (composite insertion and substitution events) smaller than the length of a short-read sequencing alignment. The output for many variant callers is a file called a VCF file. VCF stands for variant interchange format.
6. Examine the VCF file in your results (click on the eye icon to view its contents). Detailed information about VCF file content is available here: <https://samtools.github.io/hts-specs/VCFv4.2.pdf>
7. What does tool SnpEFF do? SnpEff is a variant annotation and effect prediction tool. It annotates and predicts the effects of variants on genes (such as amino acid changes).

Viewing VCF file results in a genome browser:

In order to view a VCF file in GBrowse, it first has to be converted to a format that GBrowse can understand like BigWig. To do this follow these steps:

1. Click on the edit attributes icon on the FreeBayes VCF output file.
2. In the central window click on the 'Convert Format' tab.
3. Next select the 'Convert BED, GFF or VCF to BigWig' option and click on the 'Convert' link.
4. Notice a new step will appear in you history for the conversion step.

```
8: FreeBayes on data 7 (variants)
3,511 lines, 56 comments
format: vcf, database: PiroplasmaDB-32_BmicrotIRI_Genome
display with IGV local
1. Chrom 2. Pos
##fileformat=VCFv4.1
##fileDate=20170617
##source=freeBayes v0.9.21-19-gc003c1e
##reference=/mnt/galaxyIndices2/genome
##phasing=none
##commandLine='freebayes --bam localba'
```

Attributes Convert Format Datatype Permissions

Convert to new format

Convert VCF to BGZIP

Convert VCF to VCF_BGZIP

Convert Vcf to tabix

Convert BED, GFF, or VCF to BigWig

Contents of this dataset converted to a new format.

- Once the conversion is done, you can click on the view in GBrowse link to go to the appropriate EuPathDB website and view variant locations.

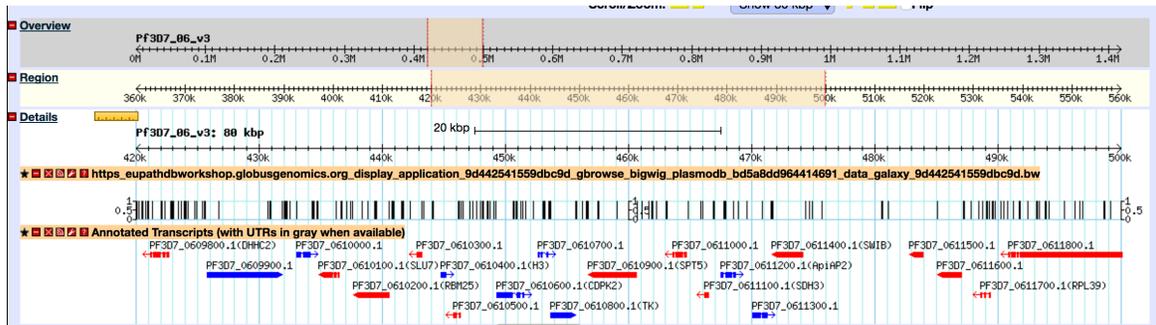
20: Convert BED, GFF, or VCF to BigWig on data 14

758.2 KB

format: **bigwig**, database: **PlasmoDB-29_Pfalciparum3D7_Genome**

[Display in PlasmoDB GBrowse](#)

Binary UCSC BigWig file



- You can also compare two VCF files to each other. To do this you need to move the VCF files you are interested in comparing into the same history then run a tool like SnpSift concordance on the files. Click on the 'History Options' icon and select copy dataset.

Copy any number of history items from one history to another.

Source History: 11: TgRH:WT_Parent (current history)

- 1: SRR5123638_1.fastq.gz
- 2: SRR5123638_2.fastq.gz
- 3: FastQC on data 1: Webpage
- 9: FastQC on data 2: Webpage
- 13: BAM to BigWig on data 12
- 16: SnpEff on data 15
- 17: SnpEff on data 15

Destination History:

Choose multiple histories

— OR —

New history named:

Copy History Items

History

- HISTORY LISTS
- Saved Histories
- Histories Shared with Me
- CURRENT HISTORY
- Create New
- Copy History
- Copy Datasets**
- Share or Publish
- Extract Workflow
- Dataset Security
- Resume Paused Jobs
- Collapse Expanded Datasets
- Unhide Hidden Datasets
- Delete Hidden Datasets
- Purge Deleted Datasets
- Show Structure
- Export Citations
- Export to File
- Delete
- Delete Permanently
- OTHER ACTIONS
- Import from File

7. Select the dataset you want to move and provide a new history name if you want to put the VCF files in a new history.
8. Select the other history you want to move VCF files from.

✓ 1 dataset copied to 1 history: VCF Compare.

i Copy any number of history items from one history to another.

Source History:
12: TgRH:WT_Parent (current history) ▾

- 1: VCF Compare
- 2: B. micro Wisconsin single
- 3: imported: Unnamed history
- 4: CompareVCF
- 5: imported: Group 2 Results
- 6: Unnamed history
- 7: C. neofrmans
- 8: Unnamed history
- 9: Unnamed history
- 10: PI2000 Prudence Island, RI:...
- 11: ENU-mutant RH clone resistant...**
- 12: TgRH:WT_Parent (current history)
- 13: Plasmodium Chloroquine...
- 14: MoryzaeSNPs
- 15: Unnamed history
- 16: Unnamed history
- 17: imported: imported: Variant...
- 18: imported: Group2: Candida...

Destination History:
▾

Choose multiple histories

— OR —

New history named:

Copy History Items

9. Rename the files so you can keep track of them.
10. Find the tool called SnpSift Concordance and select it from the tools menu on the left.

snpsift

NGS: Variant Detection

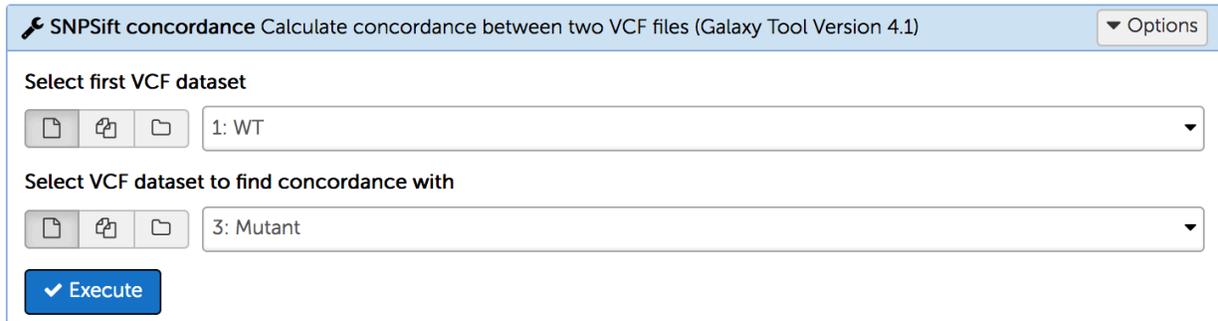
VARSCAN TOOLS

- [SnpSift Filter](#) Filter variants using arbitrary expressions
- [SnpSift CaseControl](#) Count samples are in 'case' and 'control' groups.
- [SnpSift Annotate](#) Annotate SNPs from dbSnp
- [SnpSift Intervals](#) Filter variants using intervals
- [SNPSift concordance](#) Calculate concordance between two VCF files**

Workflows

- [All workflows](#)

11. Select each of the VCF files and execute this tool.

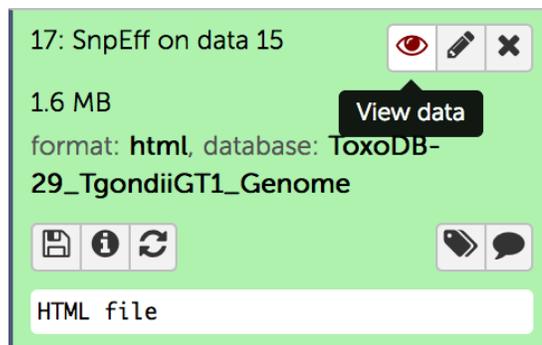


This is typically used when you want to calculate concordance between a genotyping experiment and a sequencing experiment.

12. Examine the table called 'SNPSift concordance on data 3 and data 1: stdout'
<http://snpeff.sourceforge.net/SnpSift.html#concordance>

Examining SnpEff summary:

- Click on the view icon (eye) in the SnpEff output file that has the html format.



- This will open the html file right in galaxy where you can view it.
- The header contains a short summary and information about the run and it has several major components:
 1. Summary table that warns about possible genomic annotation errors or inconsistencies identified in the reference genome. If there are many, use caution interpreting results and examine associated gff files for any issues (ex. missing feature values in gff files, incomplete gene sequences, more than one stop codon per gene, etc.).

2. Summary statistics for variant types

Number variants by type

Type	Total
SNP	114,034
MNP	12,864
INS	6,907
DEL	7,304
MIXED	2,180
INTERVAL	0
Total	143,289

Here is an example of variant calls and what they mean in terms of nucleotide changes:

Type	What is means	Example
SNP	Single-Nucleotide Polymorphism	Reference = 'A', Sample = 'C'
Ins	Insertion	Reference = 'A', Sample = 'AGT'
Del	Deletion	Reference = 'AC', Sample = 'C'
MNP	Multiple-nucleotide polymorphism	Reference = 'ATA', Sample = 'GTC'
MIXED	Multiple-nucleotide and an InDel	Reference = 'ATA', Sample = 'GTCAGT'

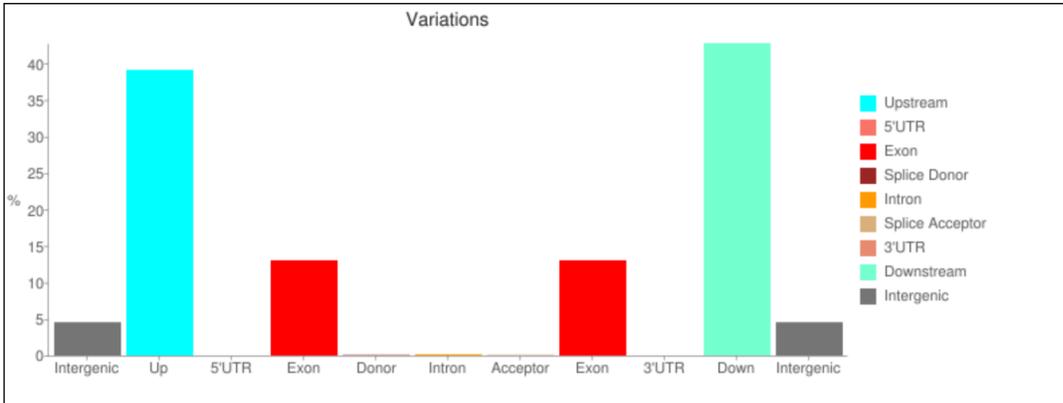
3. Statistics for the variant effects and impacts:

Number of effects by functional class

Type (alphabetical order)	Count	Percent
MISSENSE	21,588	35.949%
NONSENSE	131	0.218%
SILENT	38,332	63.832%

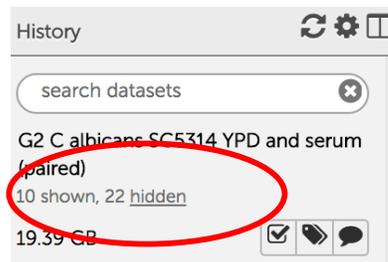
Type (alphabetical order)	Count	Percent
DOWNSTREAM	321,858	40.292%
EXON	67,505	8.451%
INTERGENIC	74,749	9.358%
INTRON	1,064	0.133%
NONE	1	0%
SPLICE_SITE_ACCEPTOR	5	0.001%
SPLICE_SITE_DONOR	4	0.001%
SPLICE_SITE_REGION	176	0.022%
TRANSCRIPT	12	0.002%
UPSTREAM	333,432	41.741%

Base changes summary. SnpEff html files provides a break down of SNPs across gene features:



The SNP workflow you are using is set up to generate certain files that will provide you with the information you can export and use further in your analysis (yellow stars).

If you select certain options they will be shown in your history. If you do not select to display these files, you can view the output by clicking on displaying the hidden files from the history menu:



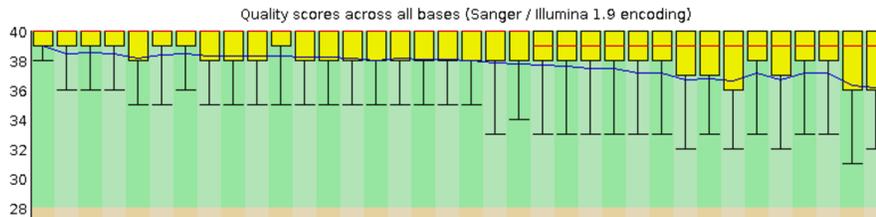
Now, let's take a look at the files generated by the workflow and steps that you can take to further evaluate them.

1. Examine sequence quality based on FastQC quality scores. FastQC provides an easy-to-navigate visual representation sequencing data quality and distribution of nucleotides per read position.

Basic Statistics

Measure	Value
Filename	SRR298691.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	4887868
Sequences flagged as poor quality	0
Sequence length	36
%GC	58

Per base sequence quality



2. Download vcf files and evaluate workflow results.

The vcf file generated by SnpEff contains information about SNPs and the genomic location.

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	unknown
CM001231	189057	.	AG	CT	787.449	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:143:0:0:143:5341:-207.887,-43.0473,0		
CM001231	483825	.	G	A	64.8756	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:4:0:0:4:146:-10.0999,-1.20412,0		
CM001231	518226	.	G	C	51.7908	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:0:0:7:276:-11.5007,-2.10721,0		
CM001231	574021	.	C	G	237.265	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:17:0:0:17:583:-39.079,-5.11751,0		
CM001231	609879	.	GAA	CAG	55.2785	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:32:8:277:22:861:-18.1711,-0.694735,0		
CM001231	1090073	.	G	T	79.4156	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:2:75:6:238:-11.5539,-1.36362,0		
CM001231	1090104	.	A	T	70.961	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:6:0:0:6:220:-12.5146,-1.80618,0		
CM001231	1153611	.	CCTC	GCTG	111.123	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:8:5:188:3:97:-9.30616,-6.1461,0		
CM001231	1159150	.	CT	GC	126.126	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:31:0:0:19:741:-29.7713,-5.71957,0		
CM001231	1159438	.	C	G	82.3312	.	AB=0;ABP=0;GT:DP:RO:Qf 0/0:47:30:1092:17:640:0,-9.53002,-3.50705		
CM001231	1159465	.	G	C	249.656	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:126:47:1770:79:3013:-53.8644,-25.2134,0		
CM001231	1159499	.	T	C	124.95	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:143:32:1167:111:4248:-76.1575,-33.4865,0		
CM001231	1181576	.	CC	TG	191.675	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:27:0:0:25:924:-41.7448,-7.52575,0		
CM001231	1293309	.	C	G	51.22	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:2:0:0:2:78:-6.92763,-0.60206,0		
CM001231	1323058	.	TT	GC	71.3001	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:6:0:0:6:223:-12.5485,-1.80618,0		
CM001231	1485397	.	A	G	3558.42	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:499:0:0:497:18671:-804.678,-149.612,0		
CM001231	1485429	.	G	A	3783.33	.	AB=0;ABP=0;GT:DP:RO:Qf 1/1:517:1:38:516:20010:-843.425,-151.978,0		

Post-processing of SNP data is normally required to make sense of thousands of SNPs and to decide which ones have biological and functional importance. Data processing can help you to extract SNP distribution and parse associated data including GeneIDs, protein-coding annotations, and effects in sequence ontology terms such as missense or synonymous variants, stop codon gain, etc. and also link changes to the genome model.

Summary

Genome	ToxoDB-29_TgondiiGT1_Genome
Date	2017-06-17 05:56
SnPEff version	SnPEff 4.11 (build 2015-10-03), by Pablo Cingolani
Command line arguments	SnPEff -i vcf -o vcf -stats /scratch/galaxy/files/008/dataset_8107.dat ToxoDB-29_TgondiiGT1_Genome /scratch/galaxy/files/008/dataset_8105.dat
Warnings	3,941
Errors	0
Number of lines (input file)	8,411
Number of variants (before filter)	8,483
Number of not variants (i.e. reference equals alternative)	0
Number of variants processed (i.e. after filter and non-variants)	8,483
Number of known variants (i.e. non-empty ID)	0 (0%)
Number of multi-allelic VCF entries (i.e. more than two alleles)	72
Number of effects	14,149
Genome total length	63,945,332
Genome effective	

SNP result visualization using Ensembl's *Variant Effect Predictor*

Ensembl provides this service for certain organisms including higher eukaryotes, fungi and *Plasmodium falciparum*.

The effect of variants on your genome of interest can be visualized using the ensembl variant effect predictor. You can do this by uploading a VCF file here:

Variant Effect Predictor for Fungi:

http://fungi.ensembl.org/Saccharomyces_cerevisiae/Tools/VEP?db=core

Variant Effect Predictor for *Plasmodium falciparum*:

http://protists.ensembl.org/Plasmodium_falciparum/Tools/VEP?db=core

Go to the Tools section and click on the VEP link

***Note that the upload file size limit is 50MB. Filtered VCF files are smaller than unfiltered ones. **Steps to get a VCF file from galaxy and load to VEP**

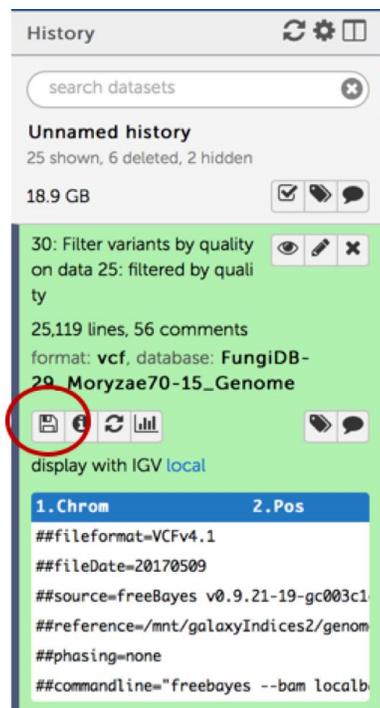
1. Click on on the save icon for the filtered vcf file. This could be any vcf file after (and including) the variant filtering step.

Tools

We provide a number of ready-made tools for processing both our data and yours. We routinely delete results from our servers after 10 days, but if you have an [ensembl account](#) you will be able to save the results indefinitely.

Processing your data

Name	Description	Online tool	Upload limit	Download script	Documentation
Variant Effect Predictor 	Analyse your own variants and predict the functional consequences of known and unknown variants via our Variant Effect Predictor (VEP) tool.		50MB*		
HMMER	Quickly search our genomes for your protein sequence.				
BLAST/BLAT	Search our genomes for your DNA or protein sequence.		50MB		
Assembly Converter	Map (liftover) your data's coordinates to the current assembly.		50MB		
ID History Converter	Convert a set of Ensembl IDs from a previous release into their current equivalents.		50MB		



History

search datasets

Unnamed history
25 shown, 6 deleted, 2 hidden
18.9 GB

30: Filter variants by quality on data 25: filtered by quality
25,119 lines, 56 comments
format: vcf, database: FungiDB-29_Moryzae70-15_Genome

display with IGV [local](#)

1. Chrom	2. Pos
##fileformat=VCFv4.1	
##fileDate=20170509	
##source=freeBayes v0.9.21-19-gc003c1	
##reference=/mnt/galaxyIndices2/genom	
##phasing=none	
##commandline="freebayes --bam localb	

Once the file is downloaded, go to the Ensembl fungi VEP page. On this page start by selecting the organism you called SNPs on from the drop down menu.

The screenshot shows the Ensembl Fungi VEP interface. The 'Species' dropdown menu is open, displaying a list of organisms. The 'Run' button is highlighted with a red circle.

Species	Options
Magnaporthe oryzae, (TaxID 242507)	Saccharomyces cerevisiae
Magnaporthe oryzae, (TaxID 242507)	Magnaporthe poae, (TaxID 644358)
Magnaporthe oryzae, (TaxID 242507)	Komagataella pastoris, (TaxID 644223)
Magnaporthe oryzae, (TaxID 242507)	Komagataella pastoris (DCA_001708105), (TaxID 4822)
Magnaporthe oryzae, (TaxID 242507)	Komagataella phaffii CBS 7435, (TaxID 981350)
Magnaporthe oryzae, (TaxID 242507)	Komagataella phaffii, (TaxID 460918)

Next click on the choose file button and select the vcf file you downloaded and click on Run.

The screenshot shows the Ensembl Fungi VEP interface. The 'Run' button is highlighted with a red circle.

Field	Value
Species	Magnaporthe oryzae, (TaxID 242507)
Name for this job (optional)	
Either paste data	
Or upload file	Browse... Galaxy30-[Filter_variants_by_quality_on_data_25_filtered_by_quality].vcf
Or provide file URL	
Identifiers and frequency data	Additional identifiers for genes, transcripts and variants; frequency data
Extra options	e.g. SIFT, PolyPhen and regulatory data
Filtering options	Pre-filter results by frequency or consequence type
Run	Run >
Clear	Clear

The job will start running and will be marked as done when finished.

5. Explore the results (refer to ensembl exercises from earlier today). For example, you can filter the results based on consequence, then sort them in the table to look at ones with High impact.

Variant Effect Predictor results

Job details | Summary statistics

Category: Count
 Variants processed: 24796
 Variants filtered out: 0
 Novel / existing variants: -
 Overlapped genes: 12537
 Overlapped transcripts: 12591
 Overlapped regulatory features: -

Consequences (all)

- splice_acceptor_variant: 43%
- splice_region_variant: 43%
- missense_variant: 3%
- intron_variant: 3%
- synonymous_variant: 2%
- 3_prime_UTR_variant: 2%
- 5_prime_UTR_variant: 2%
- regulatory_variant: 1%
- splice_region_variant: 0%
- Others: 0%

Coding consequences

- missense_variant: 55%
- synonymous_variant: 27%
- frameshift_variant: 3%
- stop_gained: 1%
- inframe_deletion: 1%
- inframe_insertion: 1%
- coding_sequence_variant: 1%
- protein_altering_variant: 1%
- start_lost: 0%

Filters
 Consequence is defined

Impact	Symbol	Gene	Biotype
High	MODIFIER	IRNA-Pseudo	EFMOG0
High	MODIFIER	-	MGG_01
High	MODIFIER	-	-
High	MODIFIER	-	-
High	MODIFIER	-	-

Results preview
 Consequence is coding_sequence_variant

Uploaded variant	Location	Allele	Consequence	Impact	Symbol	Gene	Feature type	Feature	Biotype	Exon
CM001231:209683-209683		T	stop_gained	HIGH	MGG_15994	Transcript	MGG_15994T0	protein_coding	8/8	
CM001231:79227-79227		G	synonym							
CM001231:154472-154472		C	synonym							
CM001231:195138-195138		T	synonym							
CM001231:196528-196528		T	synonym							
CM001231:197315-197315		T	synonym							
CM001231:197354-197354		C	synonym							
CM001231:197855-197855		A	synonym							
PM001951:102802-102802		C	synonym							

Region in detail
 Location: 1209633-209733
 Gene: MGG_15994

Sequence:
 209683 209684 209685 209686 209687 209688 209689 209690 209691 209692 209693 209694 209695 209696 209697 209698 209699 209700 209701 209702 209703 209704 209705 209706 209707 209708 209709 209710 209711 209712 209713 209714 209715 209716 209717 209718 209719 209720 209721 209722 209723 209724 209725 209726 209727 209728 209729 209730 209731 209732 209733 209734 209735 209736 209737 209738 209739 209740 209741 209742 209743 209744 209745 209746 209747 209748 209749 209750 209751 209752 209753 209754 209755 209756 209757 209758 209759 209760 209761 209762 209763 209764 209765 209766 209767 209768 209769 209770 209771 209772 209773 209774 209775 209776 209777 209778 209779 209780 209781 209782 209783 209784 209785 209786 209787 209788 209789 209790 209791 209792 209793 209794 209795 209796 209797 209798 209799 209800 209801 209802 209803 209804 209805 209806 209807 209808 209809 209810 209811 209812 209813 209814 209815 209816 209817 209818 209819 209820 209821 209822 209823 209824 209825 209826 209827 209828 209829 209830 209831 209832 209833 209834 209835 209836 209837 209838 209839 209840 209841 209842 209843 209844 209845 209846 209847 209848 209849 209850 209851 209852 209853 209854 209855 209856 209857 209858 209859 209860 209861 209862 209863 209864 209865 209866 209867 209868 209869 209870 209871 209872 209873 209874 209875 209876 209877 209878 209879 209880 209881 209882 209883 209884 209885 209886 209887 209888 209889 209890 209891 209892 209893 209894 209895 209896 209897 209898 209899 209900 209901 209902 209903 209904 209905 209906 209907 209908 209909 209910 209911 209912 209913 209914 209915 209916 209917 209918 209919 209920 209921 209922 209923 209924 209925 209926 209927 209928 209929 209930 209931 209932 209933 209934 209935 209936 209937 209938 209939 209940 209941 209942 209943 209944 209945 209946 209947 209948 209949 209950 209951 209952 209953 209954 209955 209956 209957 209958 209959 209960 209961 209962 209963 209964 209965 209966 209967 209968 209969 209970 209971 209972 209973 209974 209975 209976 209977 209978 209979 209980 209981 209982 209983 209984 209985 209986 209987 209988 209989 209990 209991 209992 209993 209994 209995 209996 209997 209998 209999 210000