ADVANCED BIOMEDICAL COMPUTING CENTER

Annotation, Visualization, and Impact Analysis (AVIA) Tutorial

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A plethora of information that emerges from large-scale genome characterization studies has triggered a development of computational frameworks and tools for efficient analysis, interpretation and visualization of genomic data. Functional annotation of genomic variations and the ability to visualize the data in the context of whole genome and/or multiple genomes has remained a challenging task. We have developed an interactive web-based tool, AVIA (Annotation, Variation and Impact Analysis), to explore and interpret large sets of genomic variations (single nucleotide variations (SNVs) and insertion/deletions) to help guide and summarize genomic experiments.

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AVIA Tutorial

I) What is AVIA?

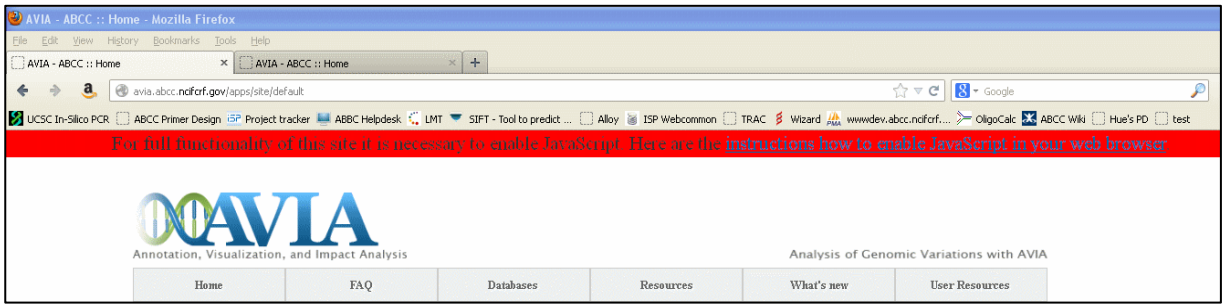
AVIA, or Annotation, Variation and Impact Analysis, is a web server dedicated to annotation of genomic variations (SNPs and InDels) found through the high-throughput sequencing. It utilizes ANNOVAR (www.openbioinformatics.org/annovar/) as the core computational framework for assigning functional impact to genomic variations. AVIA aggregates a variety of annotation databases and analysis tools for comprehensive analysis and visualization of variation data. The annotation in AVIA is gene-centric. The default source of the basic gene set is RefSeq annotation; however, Ensembl may be selected upon request.

II) What do I need to get started?

There are several workflows available through AVIA. Each workflow accepts mapped genomic variation data in one of the formats: VCF4, BED, CLC Bio, and HGVS. For training purposes of this tutorial, we will use sample data from the Riken Liver Cancer set from http://www.icgc.org.

You will also need an Internet web browser (not Microsoft Internet Explorer) with javascript enabled. You will not be able to submit to AVIA unless javascript is enabled. If you do not have javascript enabled, you will see the error message at the top of the page, as shown in Figure 1.

Figure 1. Javascript Not Enabled



As shown in the banner, if you do not know how to enable javascript, please see this tutorial at http://enable-javascript.com/. Be sure to close your browser after enabling javascript or reload the AVIA web page so that the changes can take effect.

III) File Formats

Like ANNOVAR, the preferred input format is a tab delimited **BED**-like file (http://genome.ucsc.edu/FAQ/FAQformat.html#format1). All other formats will be converted into BED-like file and used for analysis for consistency throughout the pipeline. The BED-like format has five

required fields: chromosome, genomic start, genomic stop, reference allele and variant allele. All fields after the fifth column are considered as comments and are ignored by the software, but included in the final report. A "." (dot) may be substituted for the reference allele. All variants must be the same orientation (+/+) of the genome. If headers are included, please add one line to the beginning of the text file beginning with "#". For example:

#Chr	Start	Stop	Ref	Allele	SampleInfo …

The program will report an "ERR" for annotations on any lines that do not start with the 5 required fields or does not start with "#".

VCF, or variant call format, as described by University of California Santa Cruz (UCSC), "is a flexible and extendable format for variation data such as single nucleotide variants, insertions/deletions, copy number variants and structural variants." **VCF** is a text file format (most likely stored in a compressed manner). It contains meta-information lines, a header line, and then data lines, each containing information about a position in the genome.

The following data types also accepted to AVIA, but are converted to the BED-like format described above.

1. **VCF4** format: An example of the **VCF** format from the 1000Genomes page is shown in Figure 2a. (http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-41). Comment lines beginning with "**##" a**re ignored; however, it is necessary to have the final header, beginning with the "#CHROM" to parse the data correctly shown in Figure 2a, orange box.
2. **CLCBio**: The header line from **CLC Bio's** proprietary genomics workbench output is shown in Figure 2b**.**
3. **HGVS:** The final accepted format standard is the Human Genome Variation Society (HGVS) for DNA nomenclature and is provided at http://www.hgvs.org/mutnomen/recs-DNA.html.

Figure 2. Other File Formats

a

```
##fileformat=VCFv4.1
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",t
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO                              FORMAT      NA00001
20    14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1
20    17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3
20    1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6
20    1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7
20    1234567 microsatl GTC    G,GTCT  50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4
```

b

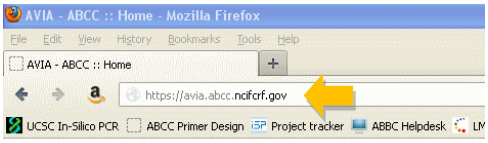
Mapping	Reference Position	Consensus Position	Variation Type	Length	Reference	Variants	Allele Variations	Frequencies	Counts	Coverage	Variant #1	Frequency of #1	Count of #1	Variant #2	Frequency of #2	Count of #2	Overlapping Annotations	Coding Region Change	Amino Acid Change

IV) Quick Start

Below, we outline the steps for you to get started using AVIA's main annotation pipeline. The Headers in Brackets following the instructions will direct you to the Section can go to get more information.

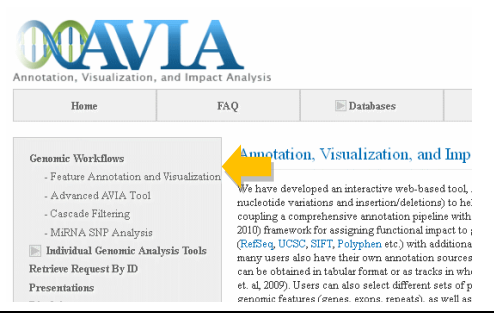
1. Go to http://avia.abcc.ncifcrf.gov on any web browser, except for Internet Explorer. [Basic Navigation]

Step 1:



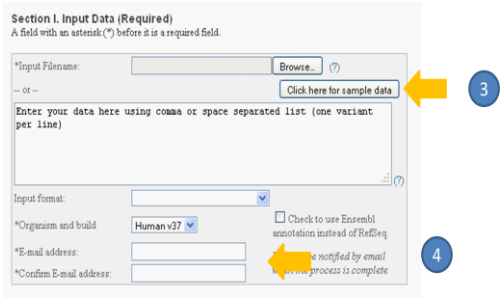
2. On the left hand menu, click on "Feature Annotation and Visualization". [Feature Annotation and Visualization]

Step 2:



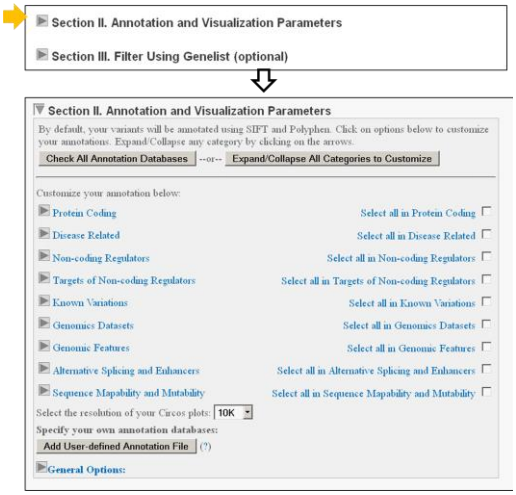
3. Under Section I, click on the button “Click here for sample data”. Sample data should appear in the text box below the button [Submission]
    a. This subset of data was taken from Riken Liver Cancer study and is the normal subtracted set of variants in BED-like format.
    b. This complete dataset is available for download at http://www.icgc.org.
    c. The “Input Format” field should automatically change to “ANNOVAR format (BED file)”.
    d. The organism and build should remain “Human v37”.
4. Enter your email address and confirmation email address.

Steps 3&4:



5. Expand Section II by clicking on the section header “Annotation and Visualization Parameters”, if it is not already expanded. [Submission | Basic Navigation of the Site]
6. Expand and select databases with which you wish to annotate. SIFT and Polyphen2 are selected by default. Any of the right arrows can be expanded to show more options. [Submission]

Steps 5&6:

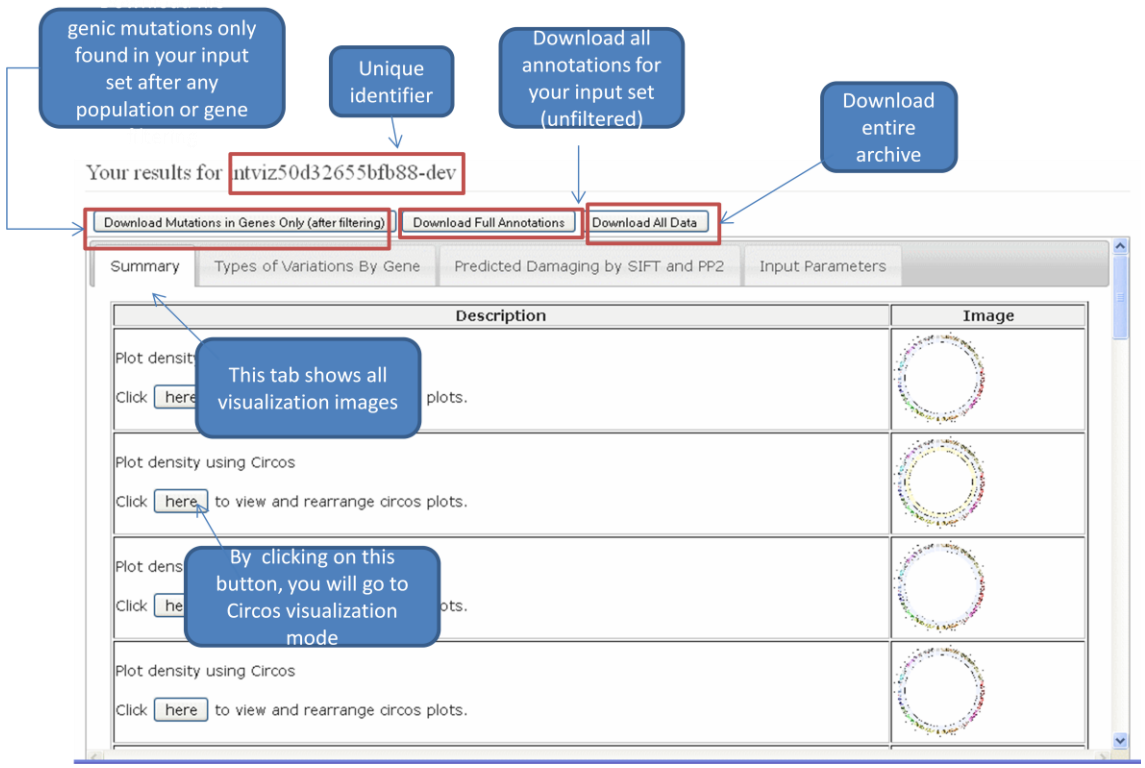


7. *Optional for tutorial: Type the letters and numbers shown in the “Captcha” box.* [1) Submission]
8. Optional for tutorial: Read and check the disclaimer. [1) Submission]
9. Click “Submit”.

Steps 7-9

View Results. By default, AVIA does not process anything using the sample data. You must change the sample data input in order to run the full pipeline. [Viewing Results on the Web]

Step 10:



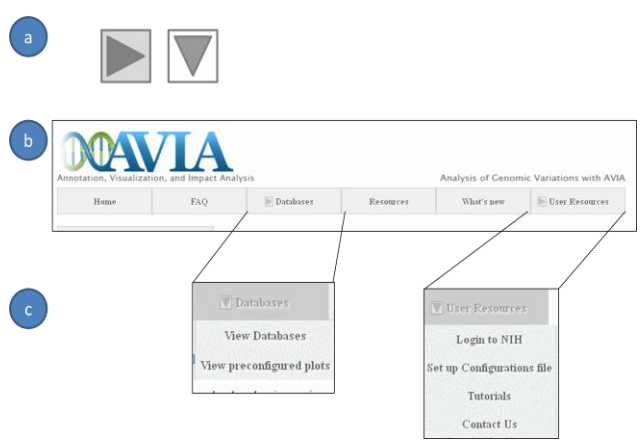
V) Basic Navigation of the Site

Figure 3. Navigation Basics

The AVIA default page is shown in Figure 3. You can always return to this page by clicking on the "AVIA" logo at the top left corner (Figure 3a) or on the Home tab (Figure 3b, first tab). Along the top, outlined in orange is the "User Navigation" Panel (Figure 3b). These tabs are user specific tools and are helpful to find useful information about our site. On the left side of the page is the AVIA Tools panel (Figure 3c). This is where the users will choose options to run the AVIA workflows using their data. We will get to those in the next section under Section IV Workflows.

From Figure 4a, you can see the two arrows that we are using to indicate whether a section of the website is expandable or collapsible. The dark gray right arrow indicates that the header may be clicked on and more options will appear. The dark gray down arrow against the white background indicates that the section can be collapsed, hiding some options. We will now briefly describe the User Navigation Panel, as this is where you will find many answers to your questions about the pipeline. The User Navigation Panel is shown in Figure 4b. You will notice that both "Databases" and "User Resources" tab have the right arrow icon. If you hover over that tab with your mouse, more options will appear, as seen in Figure 4c. Below, we briefly describe each tab and its sub categories.

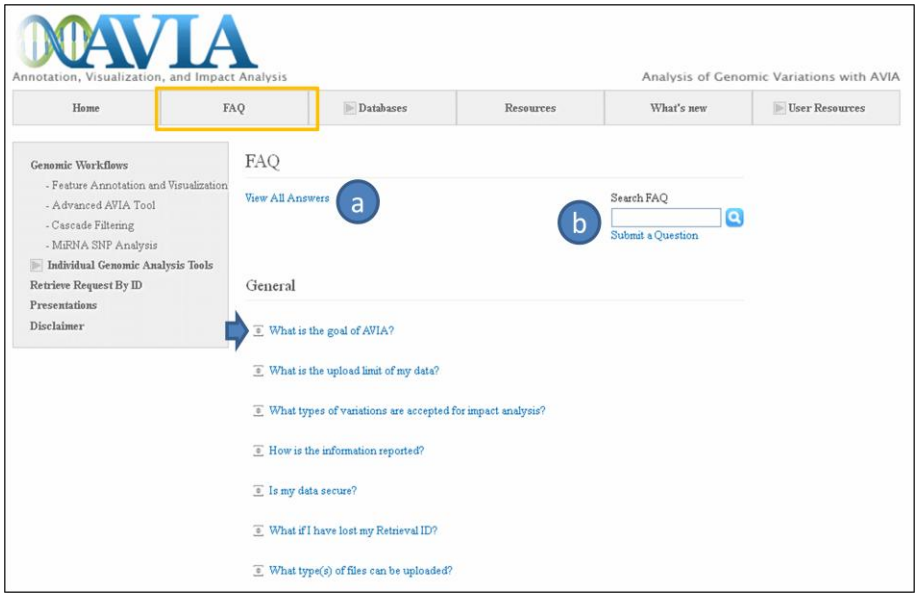
Figure 4. Additional Options



A) FAQ

By clicking on the "FAQ" tab on the User Navigation Panel, it will bring you to the FAQ section of AVIA. From this page you will see our frequently asked questions regarding the pipeline and output.

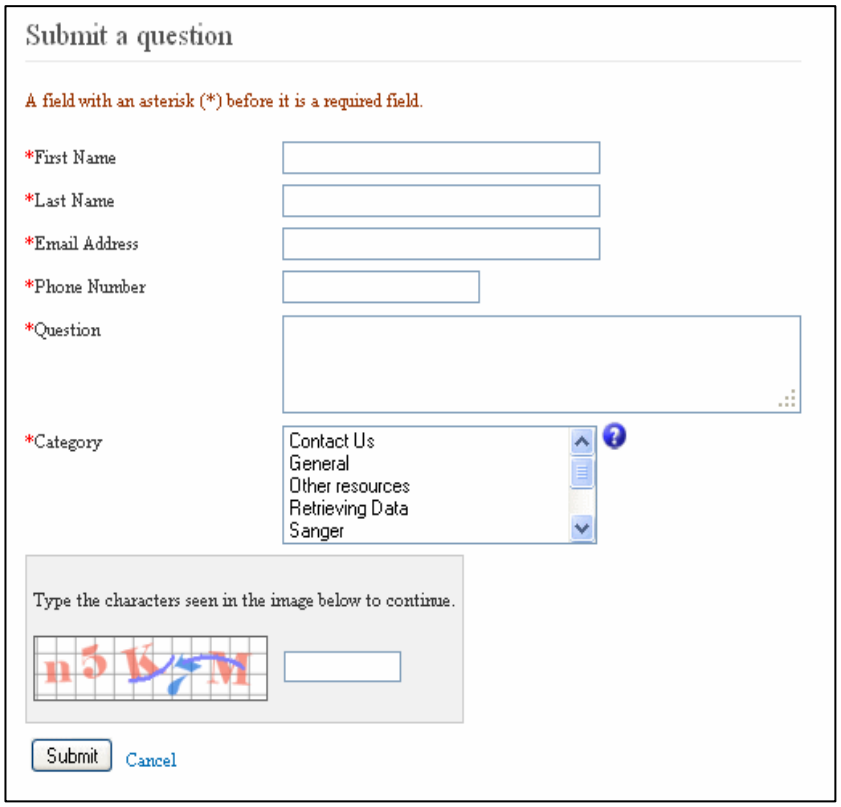
Figure 5. FAQ Section



You can choose to (Figure 5a) expand all answers to view all the answers or click on an individual question (Figure 5, blue arrow) to view the answers. You may also use the Search feature (Figure 5b) to look for a specific topic.

If you cannot find the topic that you are interested in using the Search feature, you can also click on "Submit a Question" below the search field. Using our web form shown in Figure 6, you can submit using all of the required fields. An AVIA team member will respond to your question or comment as soon as possible.

Figure 6. Submit A Question



B) Databases

Under the "Databases" tab, there are two options: "View Databases" and "View Preconfigured Plots" as shown in Figure 4c.

1) Database Information

In the "View Databases" Option, you will be able to view the most recent compiled collection of annotation databases, AVIA's Abbreviated name, the source from which data was obtained, the version or the download date and a brief description of the database with links, if available. The databases shown in Figure 7 are a snippet and may differ from the current version. Also, when you submit to AVIA, all of the annotation is available in your total archive file, which we will discuss in VII "Output Interpretation" section of the tutorial. To Expand all categories, click on the link shown in the orange box in Fig 7. The categories, highlighted by the orange arrows, can be clicked to expand or collapse the category.

Figure 7. Available Databases

Available Databases Through AVIA

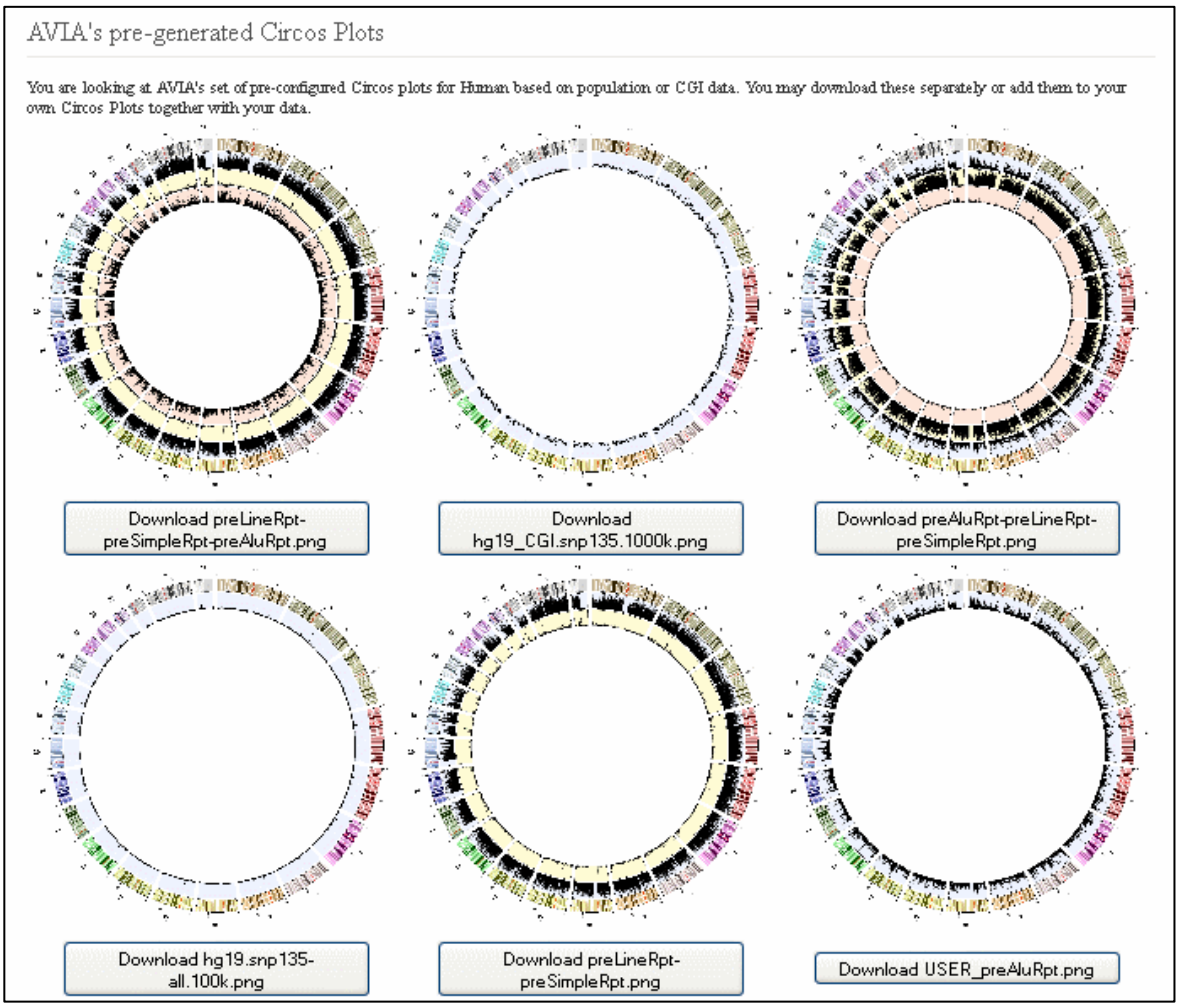
Click on each category header to expand or click here to expand all categories

Category/Database Name	AVIA Abbrv. Name	Download Source	Version	Description
Protein coding				
Combined Scores for SIFT, PP2, Phylop, LRT, MT and GERP++	ljb_all	ANNOVAR	2012Feb22	whole-exome LJBSIFT, PolyPhen, PhyloP, LRT, MutationTaster, GERP++ scores from ANNOVAR
Polyphen 2 Predictions and Scores	ljb_pp2	ANNOVAR	2011May11	Polyphen2 scores for NS SNPs with damaging prediction
Sift predictions and scores	siftv63	ANNOVAR	v63	SIFT scores with predicted damaging or tolerated based on cutoff score less than 0.05 score and a median score score greater than 3.05. Note: SIFT uses Ensembl database for annotation and may differ from the default RefSeq annotations used in AVIA.
SIFT scores	ljb_sift	ANNOVAR	2011Mar01	whole-exome LJBSIFT scores, which corresponds to 1-SIFT, which means that the closer to 1, the more damaging the mutation. Note: SIFT uses Ensembl database for annotation and may differ from the default RefSeq annotations used in AVIA.
Disease related				
COSMIC database	cosmicdb	COSMIC	hg19	Catalog of Somatic Mutations Database
OMIM	omimGene	OMIM	hg19	Online Mendelian Inheritance in Man is a comprehensive, authoritative, and timely compendium of human genes and genetic phenotypes.
Known Variations				
dbSNPv135	snp135	dbSNP	135	SNPS in the database from NCBI
Mutation Taster	ljb_mt	ANNOVAR	2011May11	Mutation Taster
Genomics datasets				
All Alt Allele Freq from 1000G Project	1000gALL_sites_2012_04	ANNOVAR	hg19	alternative allele frequency data in 1000 Genomes Project from annovar
Complete Genomics 69 Genomes	cg69	Complete Genomics	hg19	Complete Genomics
Database of Genomic Variants	dgv	UCSC	hg19	Database of Genomic Variants
GWAS Catalog	gwasCatalog	UCSC	hg19	GWAS catalog
HapMap Allele	hapmap_3_3	GATK HapMap	3.3	Data was parsed from the GATK 1.5 package. Click here to find

2) View Pre-Configured Circos Plots

For visualization, we have pre-computed several Circos Plots based on variants found whole databases or populations of data. For example, we plotted all simple repeats in one Circos plots, or all SNPs found in CGI variant data (see Figure 8; first two Circos plots). These pre-configured plots can be used in addition to your variant data plots to show the different signatures between your data and populations. We will discuss how to add these to your plots in your data in "Annotation and Visualization Parameters"> "General Options" section of the tutorial.

Figure 8. Pre-configured Circos Plots



VI) Workflows

We will now describe the "Tools Navigation Panel" as highlighted in Figure 3c on the left side panel. Four workflows will be discussed: Feature Annotation and Visualization, Advanced AVIA Workflow, Cascade Filtering and miRNA SNP analysis. The Riken Liver cancer sample data may be used in 3 out of

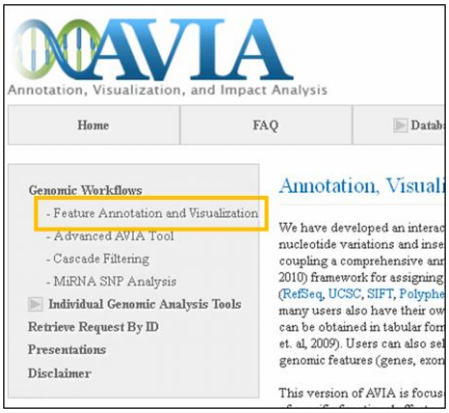
the 4 workflows to show how AVIA will report the variants differently. For the miRNA SNP analysis, there is a different usage example that you may use to run the workflow.

A) Feature Annotation and Visualization

The annotation workflow is the driving component of AVIA. We will discuss major steps (data submission, parameters selection and data retrieval) of the AVIA workflows using the Riken data as an example.

1) Submission

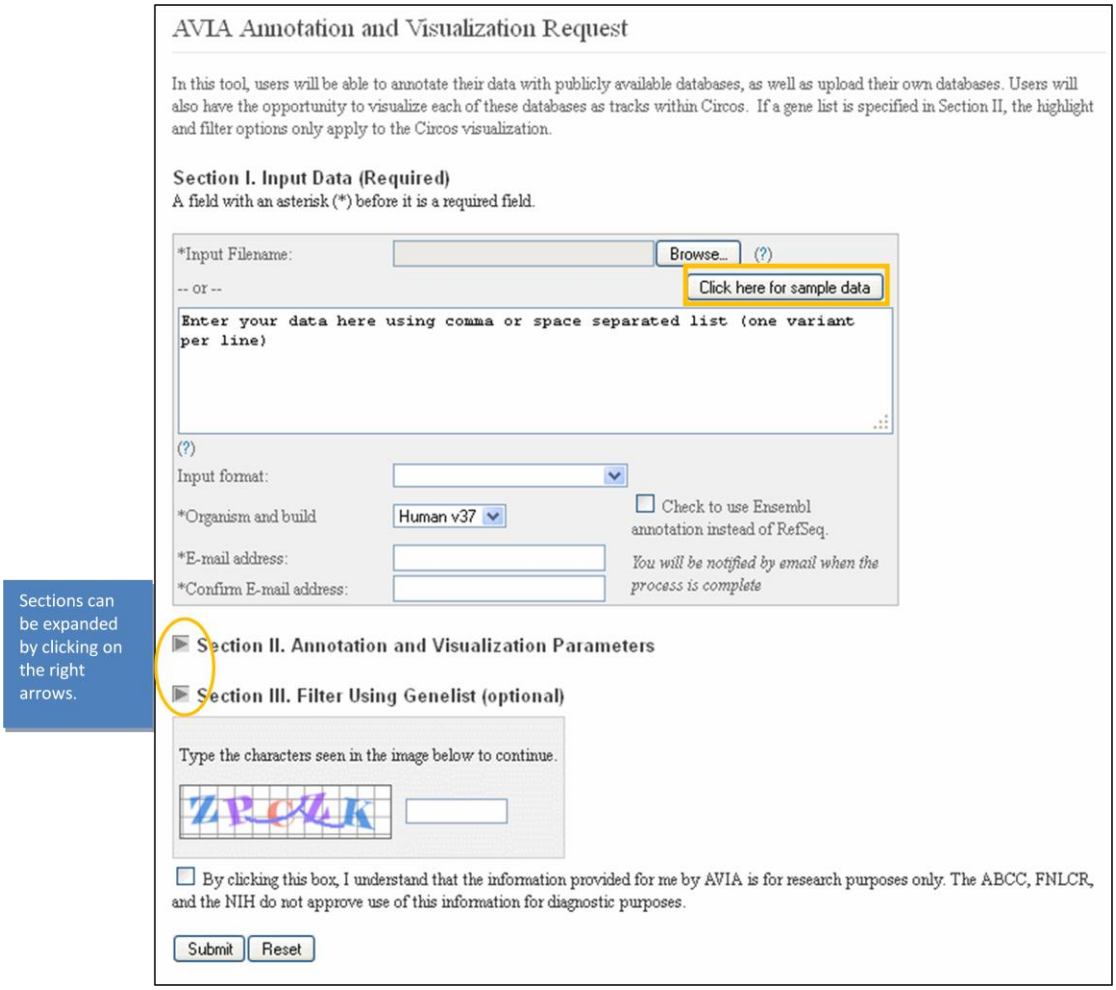
Figure 9. Navigating to AVIA Annotation Request



To begin with a new annotation request, from the AVIA home page, you can navigate to the Genomic Workflows option at the Tools Navigation Panel, located on the left side of the webpage and click on the "Feature Annotation and Visualization" as highlighted in the orange box in Figure 9. Again, please enable javascript on your web browser; otherwise, your request will not submit or will result in an error message.

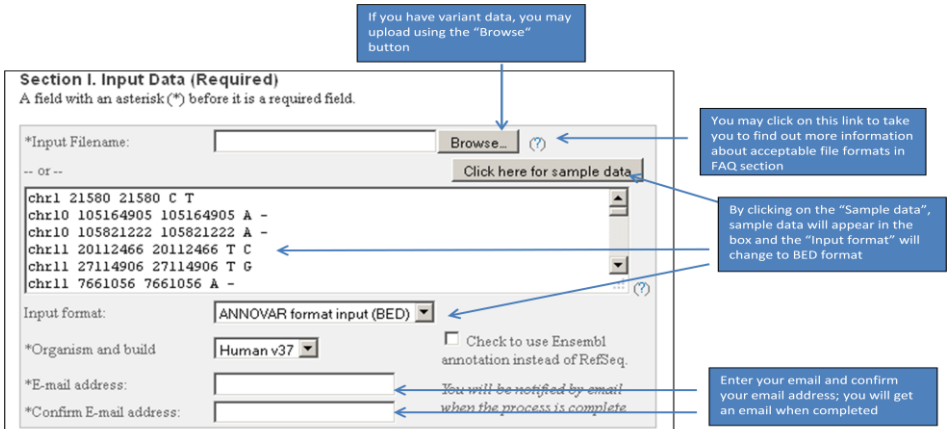
Clicking on the link will take you to the workflow page. There are three steps ("Sections") in this workflow that allow user to configure the annotation request. Section I defines the variant data, input format, origin of the data, the upload details, selection of the annotated gene features (RefSeq or Ensembl) and user's contact information. This Section is a required first step in any AVIA workflow. As described in the blue box of Figure 10 (next page), Sections II and III, clicking on the right arrows will expand the section. Section II identifies the annotation and visualization parameters of the request and Section III accommodates for gene-list focused annotation including user-supplied lists of genes and pathways. Below are some details of each of the three sections.

Figure 10. Annotation and Visualization Request Page



Section I. The input data can be submitted through the text box form by "Copy-Paste" or the input file with the data can be uploaded following the procedure: (i) click on the "Browse" button and navigate to your file; (ii) select your file using the browser and click "OK". Your path and file should appear in the text box next to the "Browse" button.

Figure 11. Input Data Section



TUTORIAL: If you do not have any data, you can still follow along by clicking on the "Click here for sample data" button, highlighted in orange, as in the Figure 11. Variation data will appear in the box or in the text box where you uploaded your file. For the sample data, the "Input format" will be changed to the "ANNOVAR format input (BED)".

If you upload a VCF4 file or any other format, you must change the "Input format" or AVIA will error and will not run. Remember that all the formats will be converted to BED-link format. You may also click on the "(?)" next to the browse button to learn about input formats or visit Section III "File Formats" in this tutorial.

Next you should select an organism, genome assembly version applied to your data and basic annotation source you wish to use.

We currently support RefSeq Human genome references (v36 and v37) as the default annotation and Ensembl as an option (v63). If your input coordinates are an older build, please note that we will use UCSC's Liftover to convert the coordinates to the newest build. Your original coordinates will be retained in the final report. Complete Section I by filling in your email address.

TUTORIAL: Please use "Human v37" as the Organism and Build. Do not check Ensembl box.

Figure 12. Annotation and Visualization Parameters

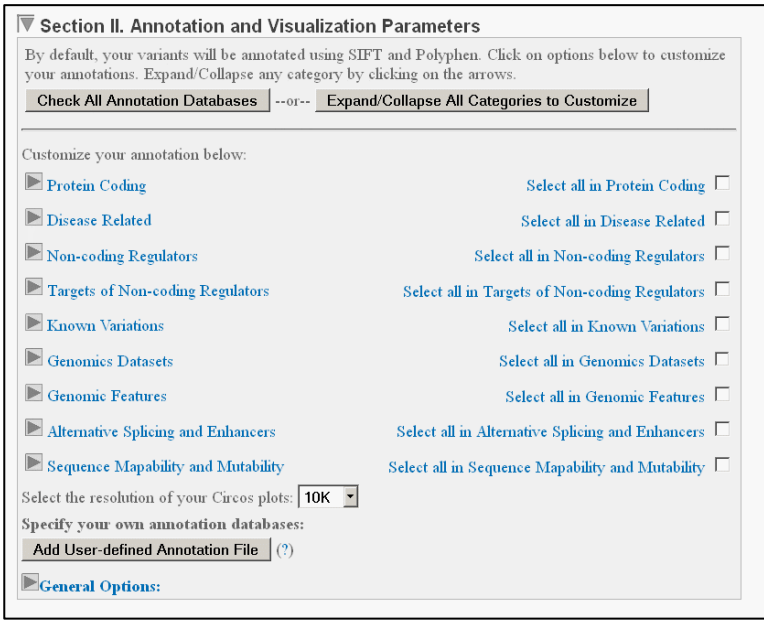
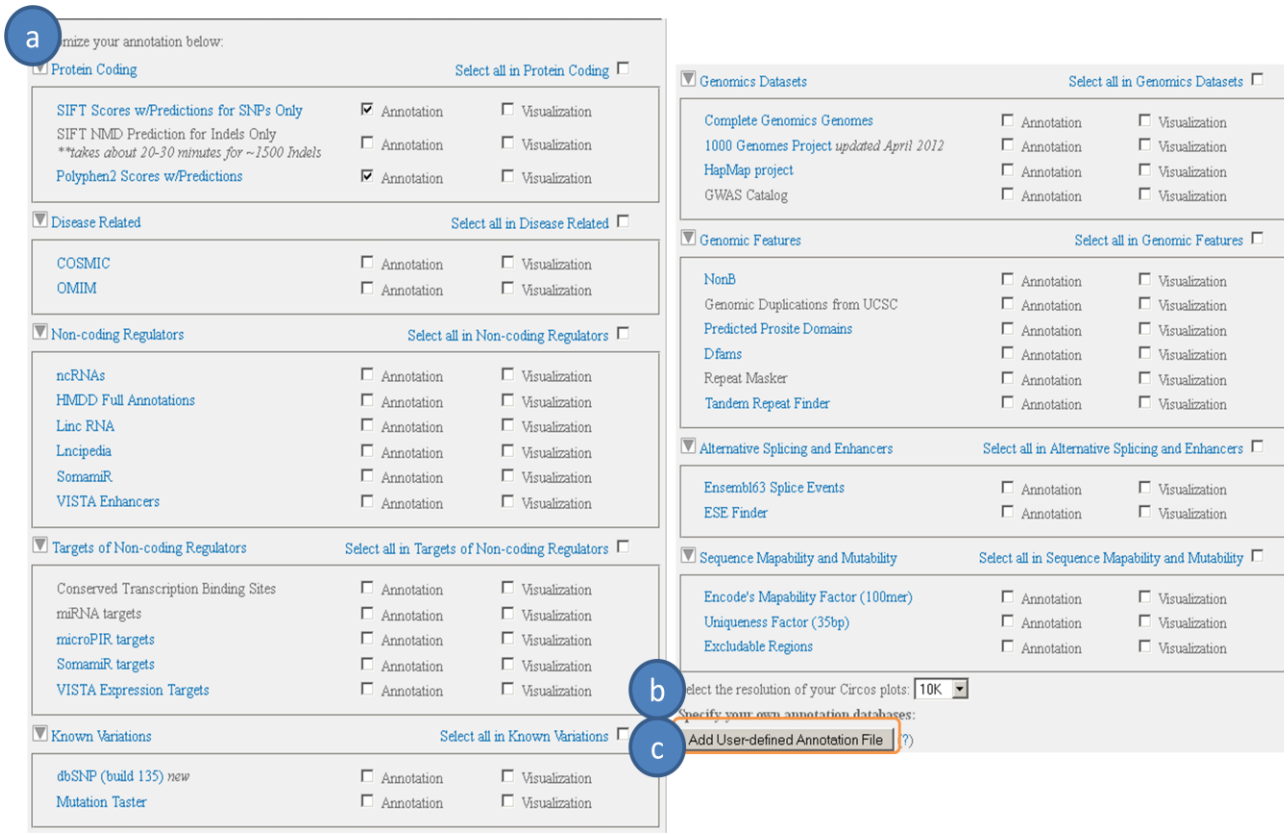


Figure 12 shows a list of publicly available databases we put together for annotation of the variations data, described in more detail on our webpage in Figure 7. Figure 12 is the default screen of the Annotation and Visualization section when you first arrive on the page. By default, the "SIFT Scores w/Predictions" and "Polyphen2 Scores w/Prediction" Annotation buttons are checked. You may click on the ▶ icon to expand or ▼ collapse the headers. You can also select all annotations by clicking on the "Check All Annotation Databases" button at the top of the section. To expand/collapse all of the categories, click on the button labeled "Expand/Collapse All Categories to Customize".

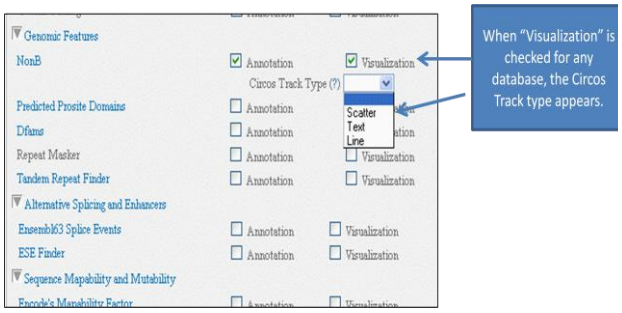
Figure 13 is the expanded version of the page. The list of the databases in the screenshot may be different than the current version; however, it illustrates two options provided by AVIA for each database in the collection: "Annotation" and "Visualization". Checking on "Annotation" box will result in additional annotation column reflecting the database content, if it exists. The "Visualization" feature can be run in addition to the "Annotation" and will lead to generation of the data track by Circos (http://www.circos.ca/).

Figure 13. Expanded Parameters for Annotation and Visualization



Once you checked the "Visualization" option, you may then select the "Circos Track Type" (Figure 14) from the drop down box. You will only have a limited track types due to the database. For example, sparse data, such as Damaging SIFT or Polyphen Scores will be plotted using scatter or dots, while dbSNP can be plotted using lines or scatter. Circos tracks corresponding to particular databases

Figure 14. Circos Types

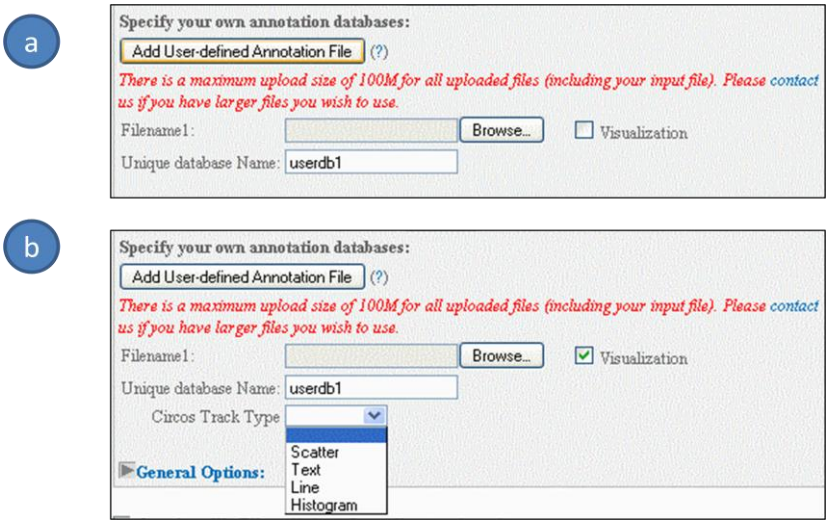


show percentage of genic variations per specified frame of the genome bin. The bin can be selected by the “Select the resolution of your plots” drop down menu found in the previous figure 13b.

If “Add User-defined Annotation File” was selected in Figure 13c, users may upload their own annotation data. By clicking the button (Figure 15a), options to upload and choose visualization options, and naming your database appear. You may choose to upload up to 10 databases for annotation. If you wish to plot a Circos plot using this database, click on the “Visualization” checkbox next to the “Browse” button, and “Circos Track Type” will appear as shown in Figure 15b. The “Unique database Name” is a field which can be used to replace the header in the AVIA output (see “Headers” in Section V Output Interpretation). This MUST be a unique name and cannot have any spaces or special characters (spaces, dashes, underscores, parentheses, brackets, ampersands, pound signs, punctuations, etc) in the name.

These details will appear each time the “Add User-defined Annotation File” button is pressed. Please note that you should first click to add the number of databases desired before filling in the details of each database.

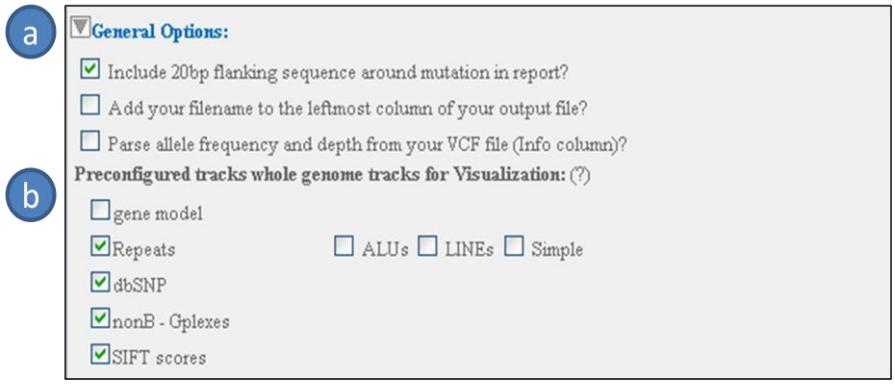
Figure 15. User supplied Annotations



At the very bottom of Section II, there are several formatting options available as shown in Figure 16. These options pertain to general options, such as adding flanking sequence to the output file, adding original filename to the leftmost column of the output file and retrieving the allele frequency information from the VCF file. If the latter is selected and you submitted a valid VCF file, it will generate a column with the "AF=" and "DP=" tags in the info column of your VCF file. This option will be ignored if it is checked and you did not submit a VCF file.

Figure 16b refers to visualization of the preconfigured whole genome tracks on your results page. You may current view this data on http://aviadev.abcc.ncifcrf.gov/apps/site/successful_viz/?id=preconfigured_plots or by selecting "Databases"> "View preconfigured plots" on your User Navigation Panel at the top of any AVIA page. From that page however, the data cannot be integrated with user variants results. To add it to your results so they can be integrated, you must select them from this menu. This option is useful for your rearranging Circos Plots after your variants have been annotated and can be useful in comparing whole population datasets to your variant data.

Figure 16. General Options for Annotation and Visualization and Cascade Filtering



The next section (Filter By Gene) allows users to focus (reduce) the annotation and visualization to specific set of the genic features. Figure 17 identifies 4 options of this section as denoted in the blue circles, which we describe below:

1) Filter vs. Highlight: This option refers to the Circos "text" track type visualization. You may wish to highlight specific genes in the variant set in such a way that your genes of interest (e.g in your gene list) will be colored red, and the rest of the mutations in genes will be colored black. Or you can choose to filter, which means only the genes in your desired gene list will be displayed, if there is a mutation in that gene.

2) Count by Gene vs Count by Variant: This option refers to how you wish to count the number of variants per bin. A bin will be defined in the "Annotation and Visualization" Section of the input parameters. If you wish to count each mutation regardless of whether it is in the same gene or not, choose, count by variants. In this option, you can have count for each bin mutation. If you wish to count mutations with genes, then choose count by

genes. This will mean that the gene mutation will count only once. This affects the frequency of the data being represented in the Circos plot.

Note: As you change from "Filter" to "Highlight" or "Count by genes" to "Count by variants" or vice versa, the description to the right will change as shown in the second box with "Highlight" and "Count By Variants" selected.

3) Use Pre-formatted Gene Lists: This option allows you to pick a gene list based on certain pathways. You may pick one or more gene lists in the option window. The gene lists are named for the source of the gene list. Your variants in these genes will be shown in the final reports. If you wish to select multiple gene lists, hold down the "CTRL" key on your keyboard and click on each of the gene lists preferred.

4) Upload your own gene list: This option allows you to upload your own gene list, one gene per row. If you have synonyms for those genes, it is also good to include those as well to ensure that all genes that you wish to be displayed are included in your final dataset. Like the previous option, this will filter for genes in your gene list and only display those requested.

Figure 17. Gene List Filters Options

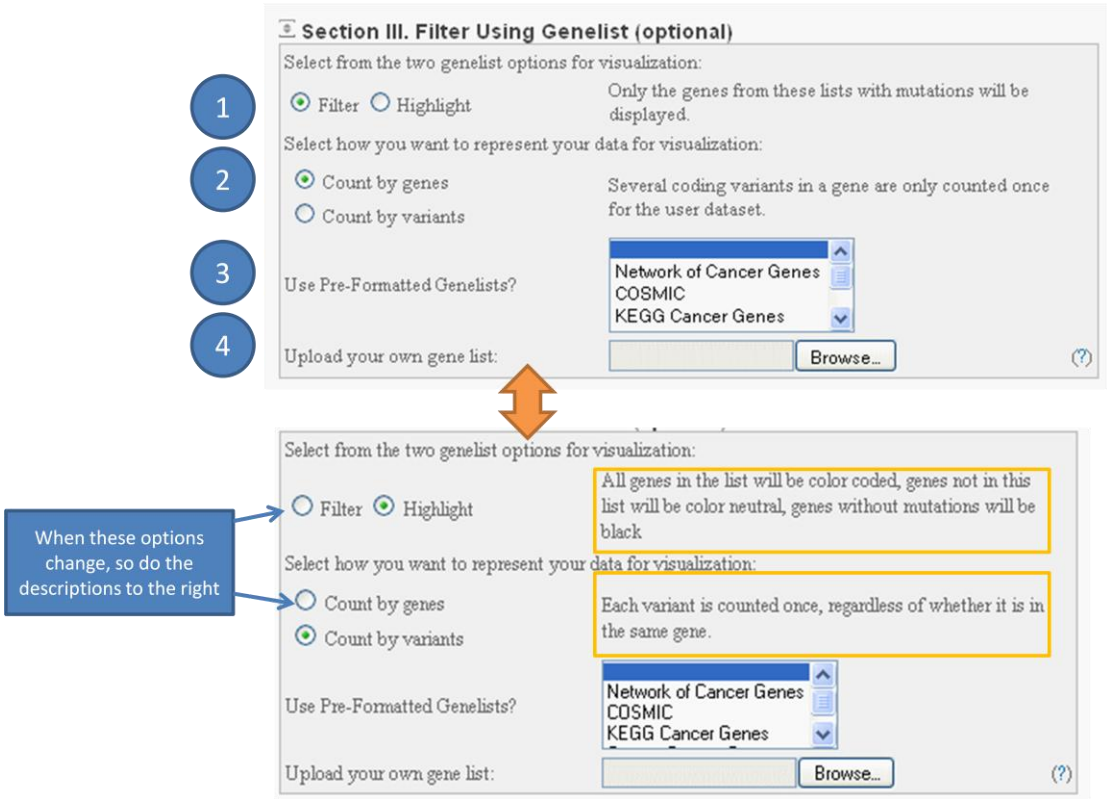
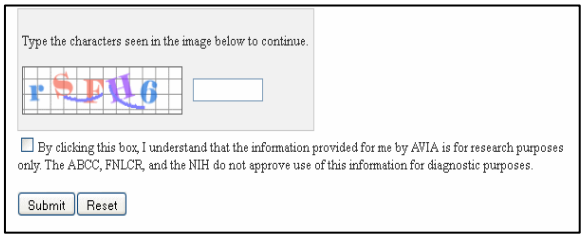


Figure 18. Captcha and Disclaimer

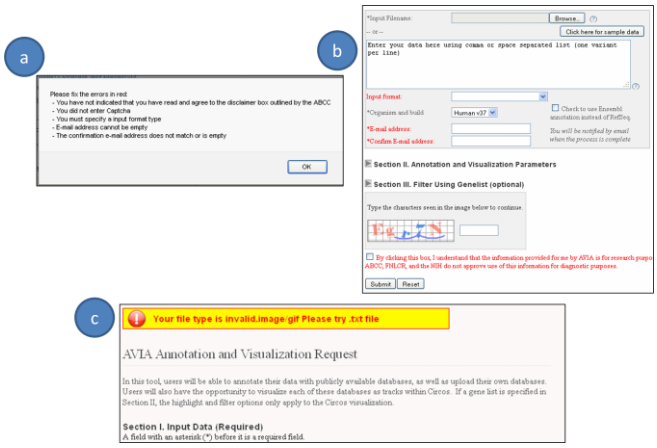


To complete your AVIA submission request, you must enter the "Captcha" in the last grey box and read and check the disclaimer acknowledgement before clicking on the "Submit" button, shown in Figure 18.

If there are any errors with your submission, a pop-up box will appear with the list of errors detected and the errant Sections on the webpage will be highlighted in red font (Figure 19a,b).

If there is a problem with your file upload(s), AVIA will provide an error message in a yellow banner as shown in Figure 19c. There is an upload limit of 200MB per file. You will get this error if your input file is too big or not the correct file format, as determined by the input file's suffix. In the example in Figure 19c, an invalid image file was uploaded.

Figure 19. Submission Errors



2) Data Retrieval

Results retrieval is a multi-step process which begins with processing page and then results in the "Results" page. Here we discuss what happens after you click the "Submit" button and how to retrieve data. The results pages will be discussed in Section VI Output Interpretation.

a) Processing

If you have successful submitted your request, you will be redirected to the status page as shown in Figure 20a. This page is updated every minute until your request is complete, up to 5 minutes. If your request completes within the allotted time, you will be automatically redirected to your results page. However, if your request takes longer than 5 minutes, you will be directed on how to proceed as shown in Figure 20b. If you choose to navigate away from this page, please take note of the unique identifier given to you , which is highlighted in orange in red font in Figure 20a. The time to process your request will depend on the size of the request and number of requests submitted to our servers.

Figure 20. Processing Page

a

Success! Your request is being processed

The current time is *Thu Jan 10 15:29:41 EST 2013*

This page will automatically refresh every minute until your results are ready. You may navigate away from this page at any time and your request will continue to process. You may come back later using your id viz50ef24b47b92d-devia on the retrieval page. You will also receive an email with a link to your results when it has completed. However, if you do not receive an email within 24 with results, please contact us using our web form.

Thank you for using AVIA.

b

Your session has timed out.

Your request is taking too long to process. Please come back later using your id viz510fd25fa83f2-dev on the retrieval page. You will receive an email with a link to your results when it has completed.

Thank you for using AVIA.

b) Email notification

You will also be notified by email at the email address provided upon completion of the request with a direct link to your results (Figure 21). Before contacting us, please check your spam folder if you do not receive a notification within 6 hours. You will have a week to retrieve your data.

Figure 21. Email notification

From: AVIA@mail.nih.gov Sent: Wed 1/30/2013 9:56 AM
To:
Cc:
Subject: Thank you for using the AVIA software

Your analysis viz510932e0c29a7-dev is now complete. You can directly link to your page by clicking below or by cutting the link below and pasting into any web browser:
http://aviadev.abcc.ncifcrf.gov/apps/site/results/?id=viz510932e0c29a7-dev

You can also retrieve other submissions by using our data retrieval page at : http://aviadev.abcc.ncifcrf.gov/apps/site/retrieve_a_request to see your results and input your id and analysis type. Your results will be stored for 1 week from the date of submission

c) Request Retrieval Page

To retrieve a request after it has timed out or if you have navigated away, click on the "Retrieve Request" from the Tools menu on the left side. If you do not have the left menu, click on the AVIA logo and it will take you to the default page. Once on the page shown in Figure 22, you can enter the unique identifier from above and the email used when you submitted your request. If completed, it will take you to your data. If not, please return later. If you have not received an email from us within 6 hours, please contact us using our web form under "User Resources"> "Contact Us".

Figure 22. Retrieve Request Page

We will discuss viewing results and interpreting the files in Section VI Output Interpretation of this tutorial.

B) Cascade Filtering (Data Reduction) Method

Previous versions of ANNOVAR's auto_annotate.pl focused on a coding-centric strategy which could potentially eliminate key variants involved in disease. Originally the idea was to be able to reduce the amount of variants to novel mutations that could be further studied. However, by eliminating key variants, like those found in dbSNP, you may also be eliminating disease causing mutations. ANNOVAR's auto_annotate.pl script used a pre-selected set of databases which could not be altered in the pipeline. Using our cascade filtering data reduction method, the user controls with which databases to filter, which data to keep, and which cutoffs (if any) to use. From the remaining set, annotation and visualization is still performed. You may choose to filter a dataset using a SIFT above a cutoff value of 0.05, and then still choose to annotate SIFT to see what the cutoff value was.

To use the cascade filtering data reduction method, click on the "Cascade Filtering" on the AVIA Tools Panel located on the left side of any AVIA page, indicated by the orange arrow and box in Figure 23.

This will take you to a page that is very similar to the Feature Annotation and Visualization page (Figure 24); however notice that "Section II" is now Cascade filtering Parameters. The section for Input Data is still required and "Annotation and Visualization Parameters Section" is also available for use.

Figure 23. Navigating to Cascade Filtering Workflow

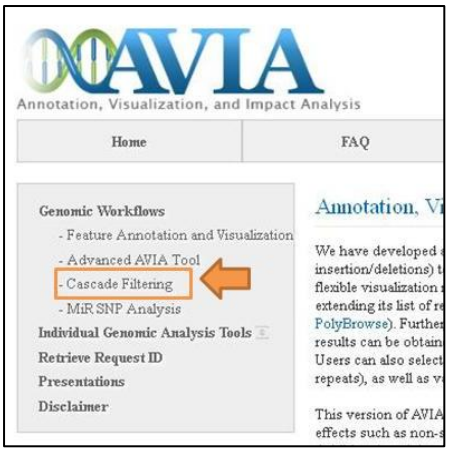
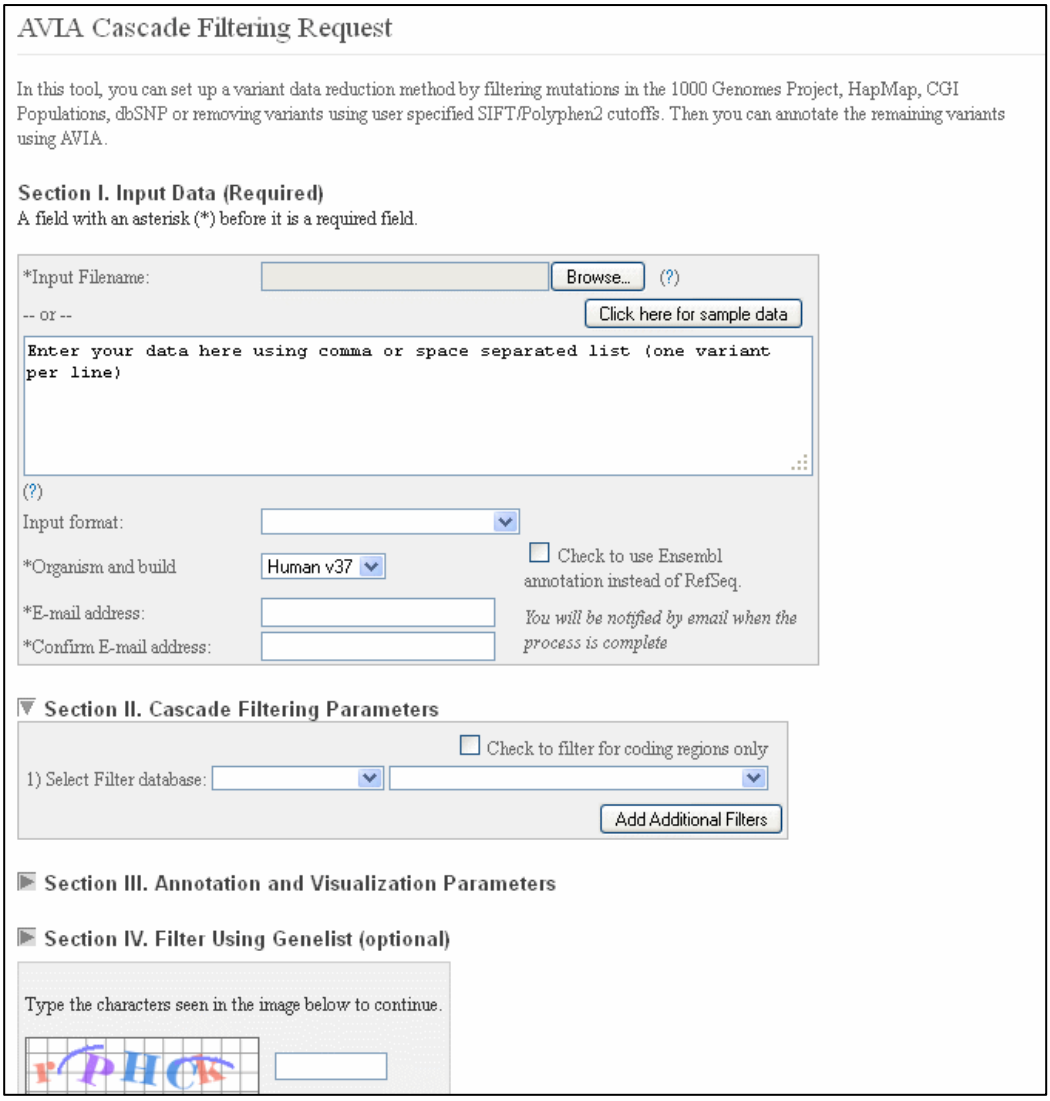
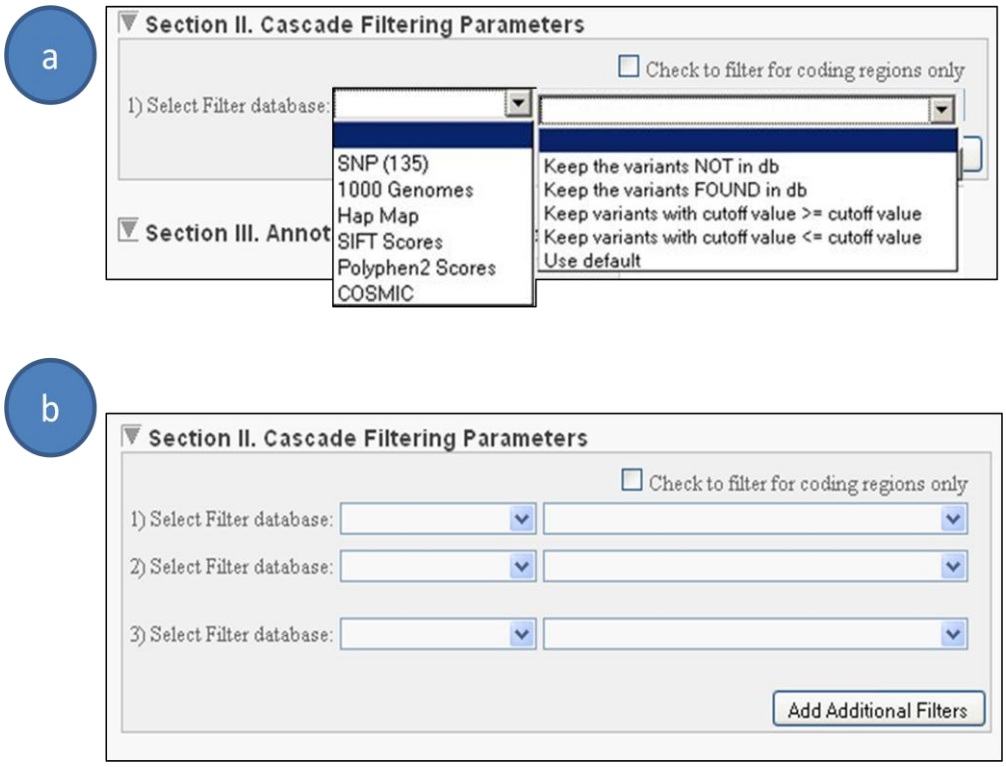


Figure 24. Cascade Filtering Workflow



In Figure 25, (a) you can choose which databases to filter with and set the cutoffs and which variants to keep and (b) you can add multiple filters up to 6 from the drop down menu list. You may then continue to select Annotations and Visualization Parameters and Gene Filter Options in Sections III and IV, respectively as shown in Figure 24 (collapsed).

Figure 25. Cascade Filtering Options

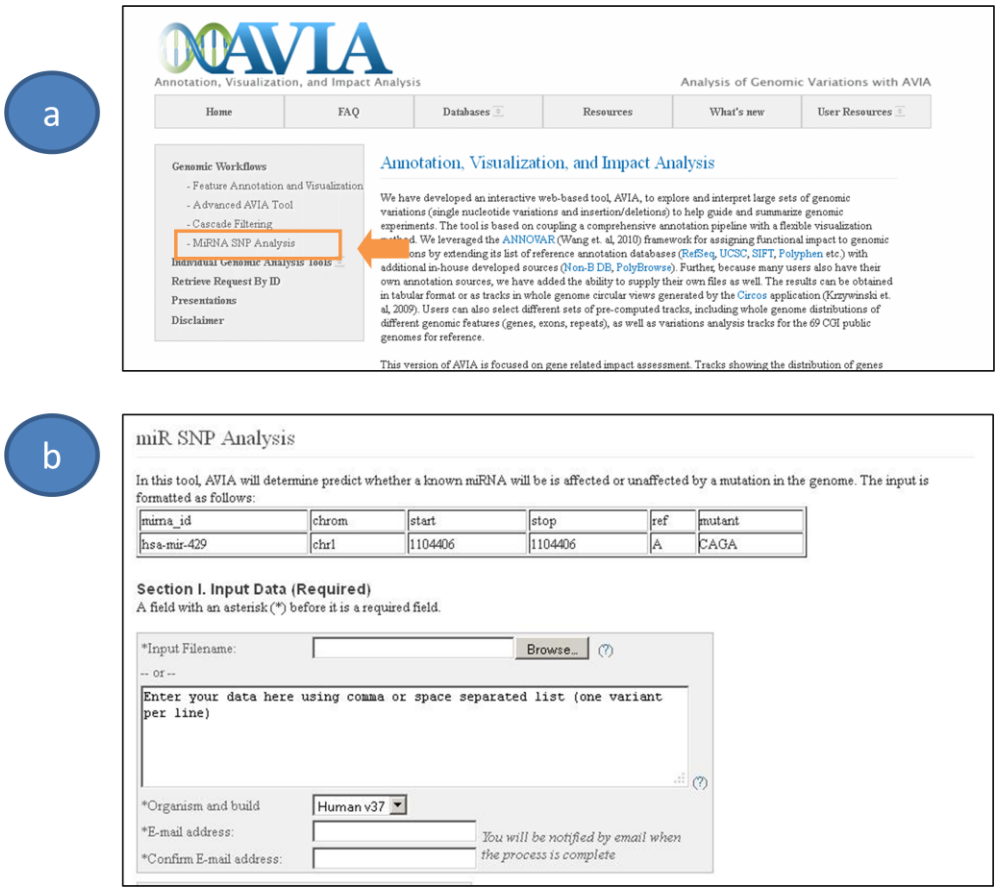


C) miRNA SNP Analysis

This pipeline is slightly different from the previous ones in that the impact analysis of miR SNPs are calculated by each individual mutation to determine whether or not the change in nucleotide will affect the seed region of the miRNA. miRNAs are very important in negative regulation in the cell and changes in the ability to bind to targets may adversely affect the interactions within the cell.

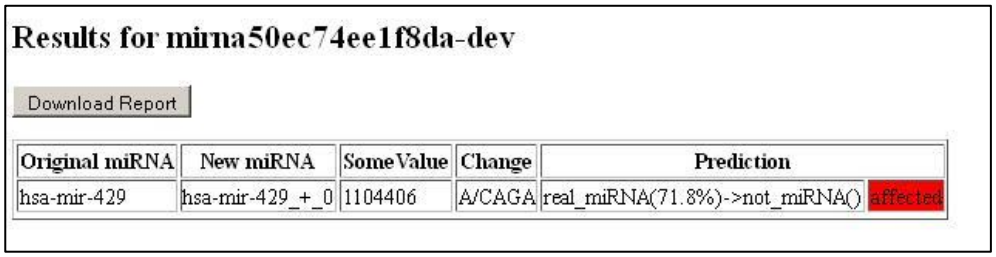
To begin, click on the "miR SNP Analysis" link on the left navigation panel as shown in Figure 25a. The input to this page requires *a priori* knowledge of the region that you are requesting impact analysis. In the Figure 25b example at the top of the page, the mirna id should be added to the beginning of each line, followed by the chromosome, start and stop positions, reference allele and variant allele. You can choose to either upload a tab delimited file or input the values in the "Cut-and-paste" box. After selecting the Organism and Build and entering Email addresses to the form, you may submit.

Figure 26. miRNA SNP Analysis



As seen in Figure 27, the output of this pipeline is a simple affected/not affected result. From here, you can see which miRNAs may be affected by the mutation.

Figure 27. miRNA SNP Analysis Results Page



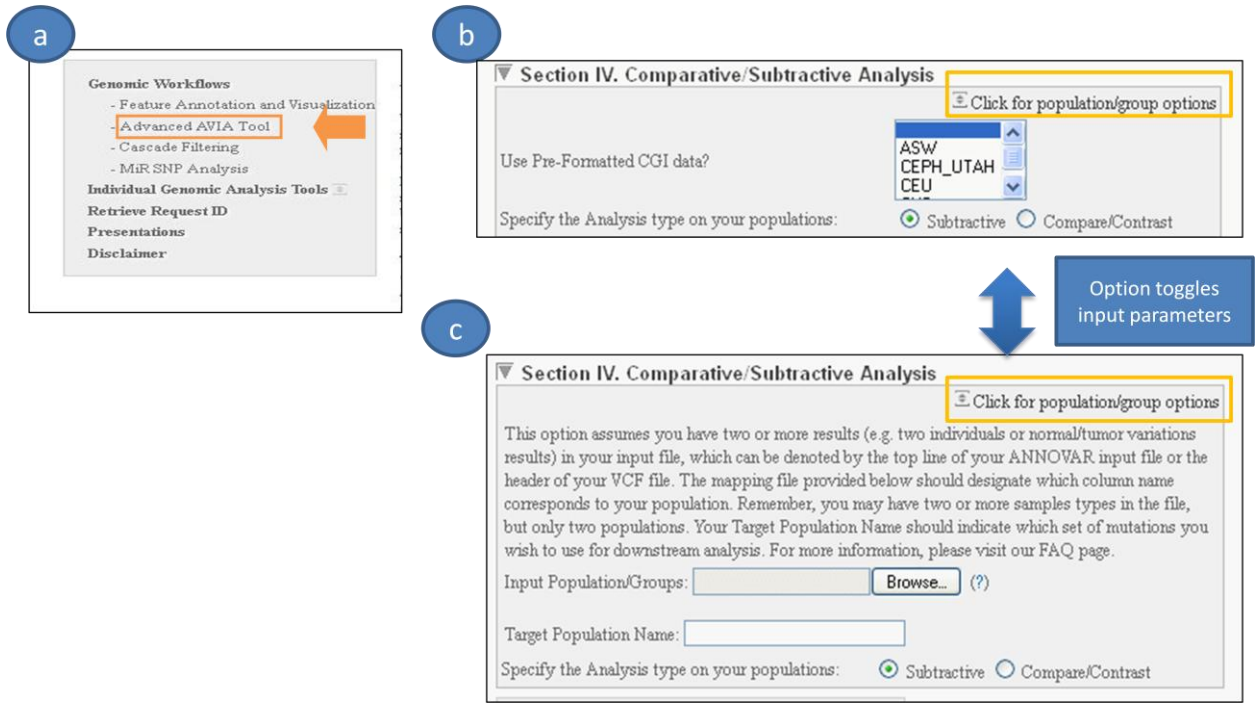
D) Advanced AVIA Workflow

This workflow has an additional component called Subtractive analysis, which is a type of filtering using related variant data. The databases we use for annotation describe a mutation in the whole genome context, e.g. dbSNP are known mutation sets across multiple populations, annotation of miRNA targets and SIFT scores to predict damaging mutations, etc are calculated based on the reference genomes. Subtractive analysis is more focused towards personalized impact assessment. We describe here how this workflow can be used to derive novel mutations within one individual or sub-population. For example, i) an individual whose variant data were sequenced from two sources, e.g tumor vs normal or ii) disease from parental lineage, e.g. child vs parental can be found. This is another way to reduce the complexity of the data, but in a more meaningful way. This workflow can also be applied to populations. If the variant data was from an individual who was sequenced and was of a different ethnicity than the NCBI reference genome, we can subtract that individual's race-specific mutations by using the allele frequencies in the Complete Genomics Population data set.

For this tutorial, we refer to "target" population as the population in which the mutations will be kept. In the examples used above, these will be the tumor sequenced mutations, child mutations or the individual's mutations. The "normal" datasets will be the blood mutations, parental and healthy ethnic populations (CEU, JPT, MXL, etc).

To begin, click on the "Advanced AVIA Workflow" on the AVIA tools panel as shown Figure 28a and fill in Sections I-III as described above. For Section IV "Comparative /Subtractive Analysis", you have the option designate the ethnicity of your normal population based on CGI populations available. You also have the option to toggle between using CGI populations as the normal population, or to

Figure 28. Advanced AVIA Workflow



upload your own data to use as normal as shown in Figure 28 b and c. The ethnicity categories were taken from Complete Genomics manual (Figure 29)

Figure 29. Ethnicity Data from Complete Genomics

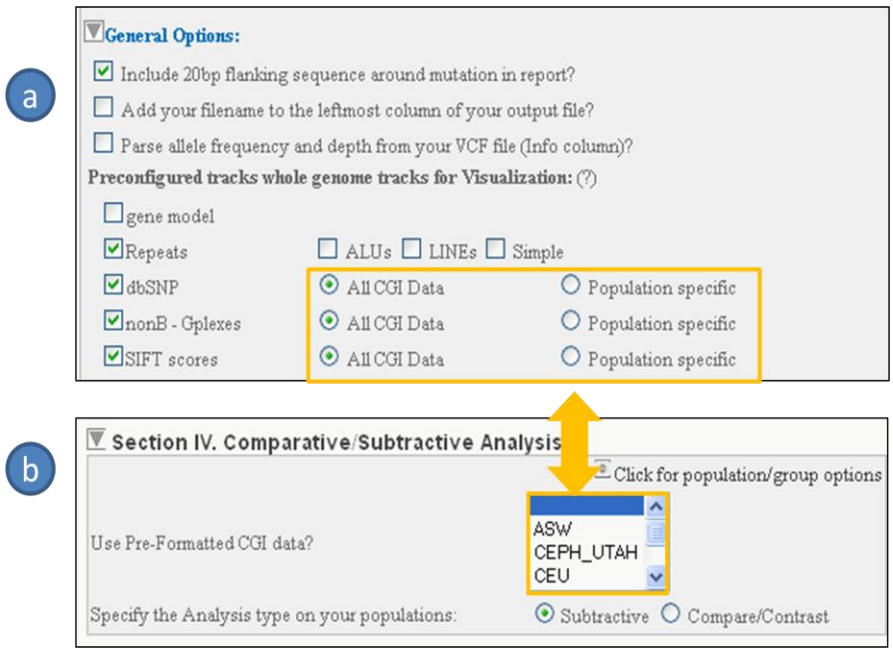
ASW: African ancestry in Southwest USA
CEU: Utah residents with Northern and Western European ancestry from the CEPH collection
CHB: Han Chinese in Beijing, China
GIH: Gujarati Indian in Houston, Texas, USA
JPT: Japanese in Tokyo, Japan
LWK: Luhya in Webuye, Kenya
MKK: Maasai in Kinyawa, Kenya
MXL: Mexican ancestry in Los Angeles, California
TSI: Toscans in Italy
YRI: Yoruba in Ibadan, Nigeria
PUR: Puerto Rican in Puerto Rico

If you choose to use your own normal population, it must be in the input VCF4 format, where the two populations are in the final 2 columns. Variants found in both your input file and your normal population will be removed from final variants set before Annotation and Visualization Parameters and Gene List Filters are applied, Sections II and III on the web page, respectively.

TUTORIAL: For the sample data, fill out Section I with the sample data, and in Section III, you can use the CGI data and pick "JPT" in the selected panel on the left. The sample data was taken from the Riken Liver Cancer data from Japanese individuals. Continue to choose the annotation that you desire and click submit. The results will be exactly the same as the results for the Annotation pipeline save for the reduction of the population mutations found in both the normal and the sample data. (Figure 28c)

There is one difference in the General Options section for the Advanced Workflow and the other workflows. As you can see from Figure 30a, now if you select dbSNP, Repeats, nonB or SIFT scores from the preconfigured visualization tracks, you can also choose to select which population for Circos visualization and comparision. This is either all CGI data combined, or population specific

Figure 30. General Options for Advanced AVIA Workflow



ethnicity. If "Population specific" is selected, AVIA will automatically use the same ethnicity selected in Section IV of the website (Figure 30b). If none are selected, AVIA will default to "All CGI Data"

VII) Output Interpretation

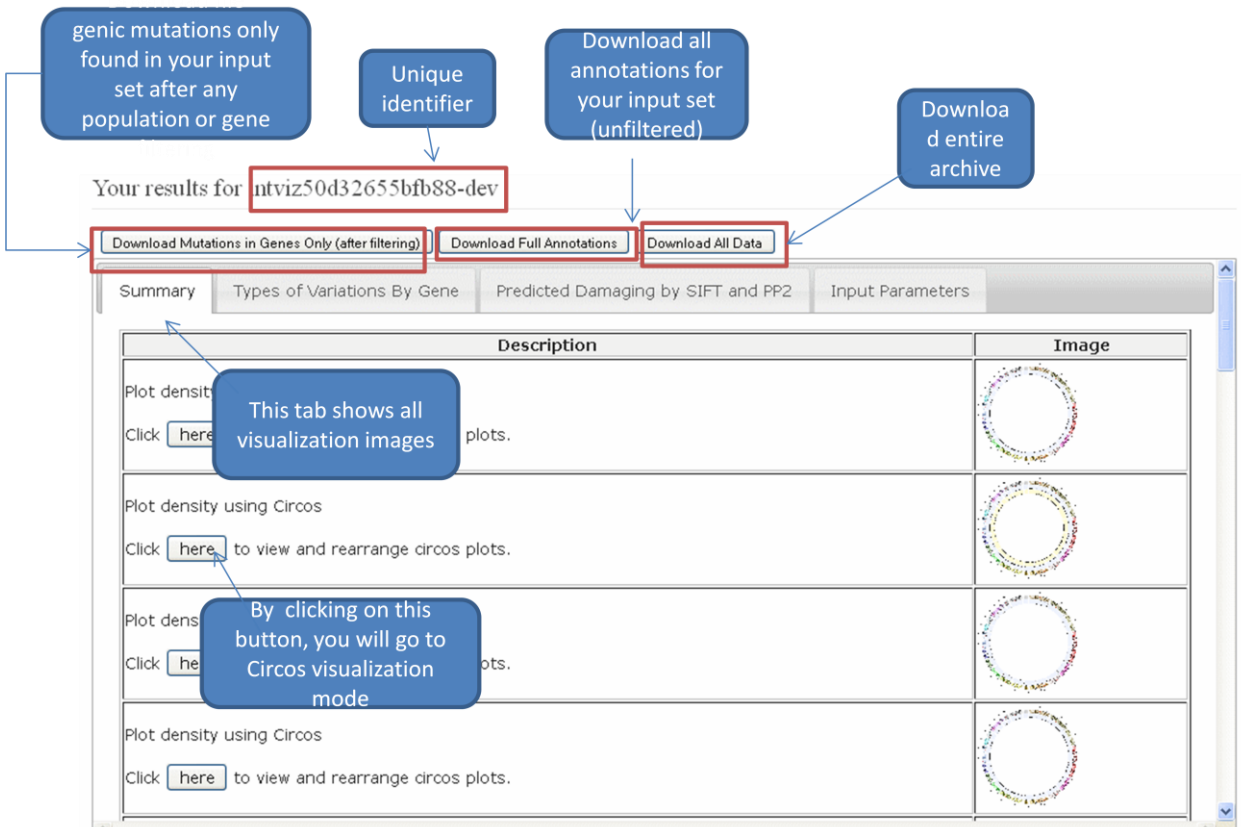
There are two ways to view the results, on the web or download to your local computer. In this section, we will describe the results as they are shown on the web and additional features that can be performed on the web. Your files will only remain on our server for 1 week after submission. At any time, you can download and save your data. In Sub section A, we will show how the files appear on the web, and in Sub section B, we will describe the final outputs.

A) Viewing Results on the Web

1) Results for Annotation and Visualization, Cascade Filtering, and Subtractive Analysis Workflows

For all workflows except for the "miR SNP Analysis" workflow, the results pages are very similar. Figure 31 is a diagram of the results landing page.

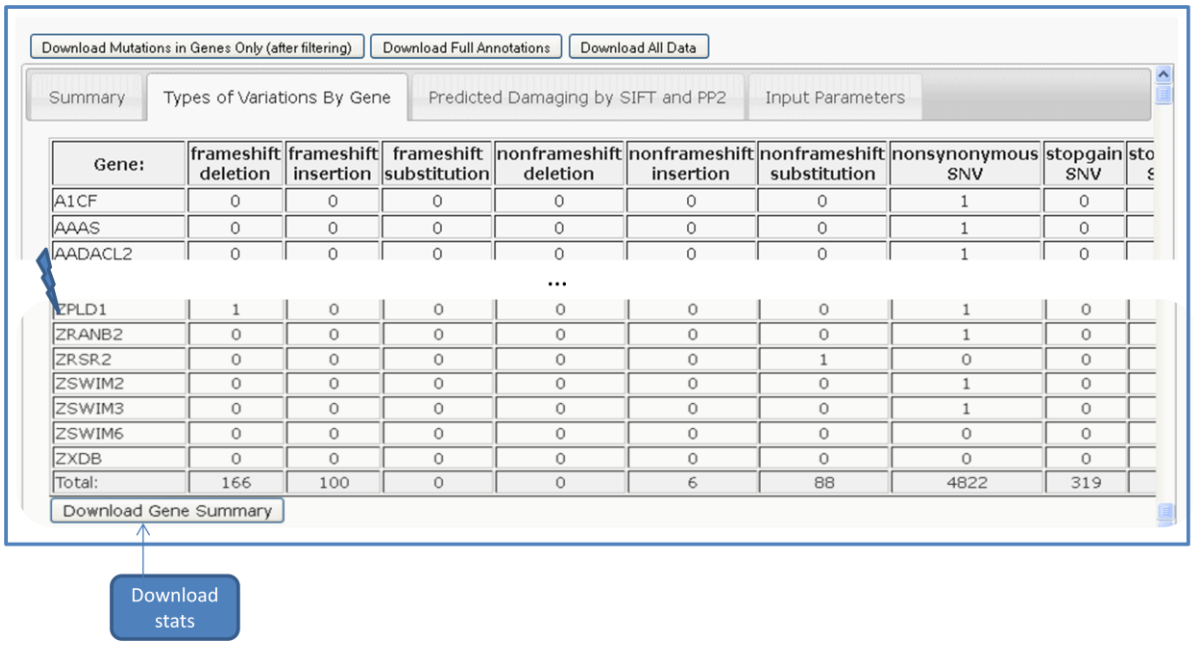
Figure 31. Results Landing Page



Tabs in Detail:

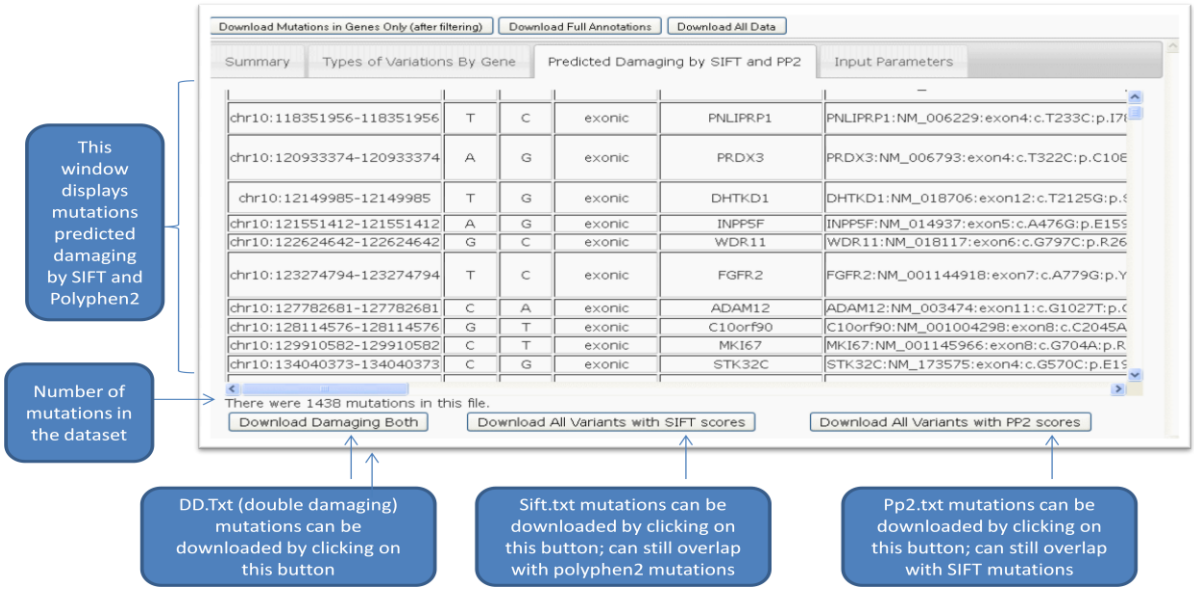
Figure 32. Variation Types By Gene Tab

This window shows a snippet of counts of mutation by type by gene. At the bottom of this tab, there is a running tally of the types of mutations types in the input dataset. These results contain all mutations by gene and mutations lost by population subtraction or gene filtering has not yet occurred.



Gene:	frameshift deletion	frameshift insertion	frameshift substitution	nonframeshift deletion	nonframeshift insertion	nonframeshift substitution	nonsynonymous SNV	stopgain SNV	sto
A1CF	0	0	0	0	0	0	1	0	
AAAS	0	0	0	0	0	0	1	0	
AADACL2	0	0	0	0	0	0	1	0	
...									
ZPLD1	1	0	0	0	0	0	1	0	
ZRANB2	0	0	0	0	0	0	1	0	
ZRSR2	0	0	0	0	0	1	0	0	
ZSWIM2	0	0	0	0	0	0	1	0	
ZSWIM3	0	0	0	0	0	0	1	0	
ZSWIM6	0	0	0	0	0	0	0	0	
ZXDB	0	0	0	0	0	0	0	0	
Total:	166	100	0	0	6	88	4822	319	

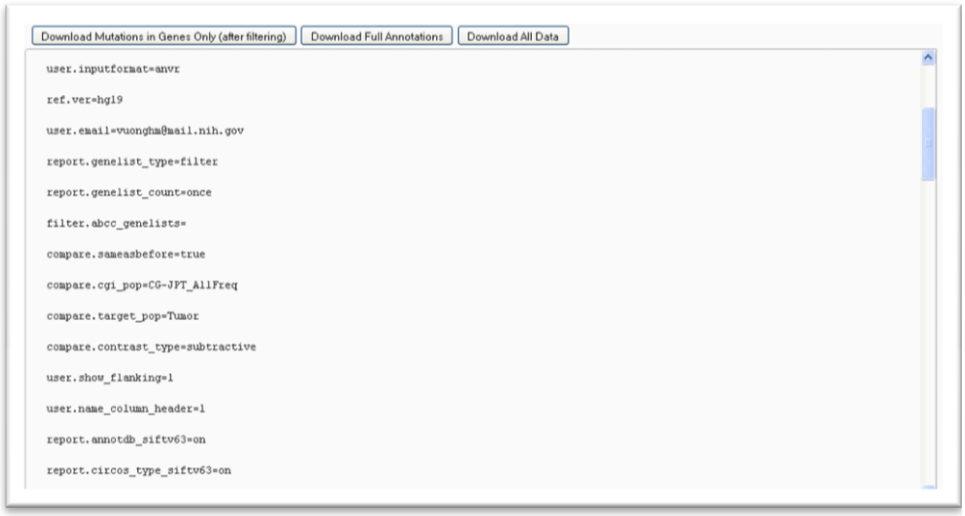
Figure 33. Damaging Mutations Tab



chr10:118351956-118351956	T	C	exonic	PNLIPRP1	PNLIPRP1:NM_006229:exon4:c.T233C:p.I7
chr10:120933374-120933374	A	G	exonic	PRDX3	PRDX3:NM_006793:exon4:c.T322C:p.C10
chr10:12149985-12149985	T	G	exonic	DHTKD1	DHTKD1:NM_018706:exon12:c.T2125G:p.
chr10:121551412-121551412	A	G	exonic	INPP5F	INPP5F:NM_014937:exon5:c.A476G:p.E15
chr10:122624642-122624642	G	C	exonic	WDR11	WDR11:NM_018117:exon6:c.G797C:p.R26
chr10:123274794-123274794	T	C	exonic	FGFR2	FGFR2:NM_001144918:exon7:c.A779G:p.Y
chr10:127782681-127782681	C	A	exonic	ADAM12	ADAM12:NM_003474:exon11:c.G1027T:p.
chr10:128114576-128114576	G	T	exonic	C10orf90	C10orf90:NM_001004298:exon8:c.C2045A
chr10:129910582-129910582	C	T	exonic	MKI67	MKI67:NM_001145966:exon8:c.G704A:p.R
chr10:134040373-134040373	C	G	exonic	STK32C	STK32C:NM_173575:exon4:c.G570C:p.E1

The Parameters tab will tell you all the information needed to recreate the analysis you just performed, including the databases run, database version, AVIA versions and the other parameters selected on the input page. Keep this with your analysis.

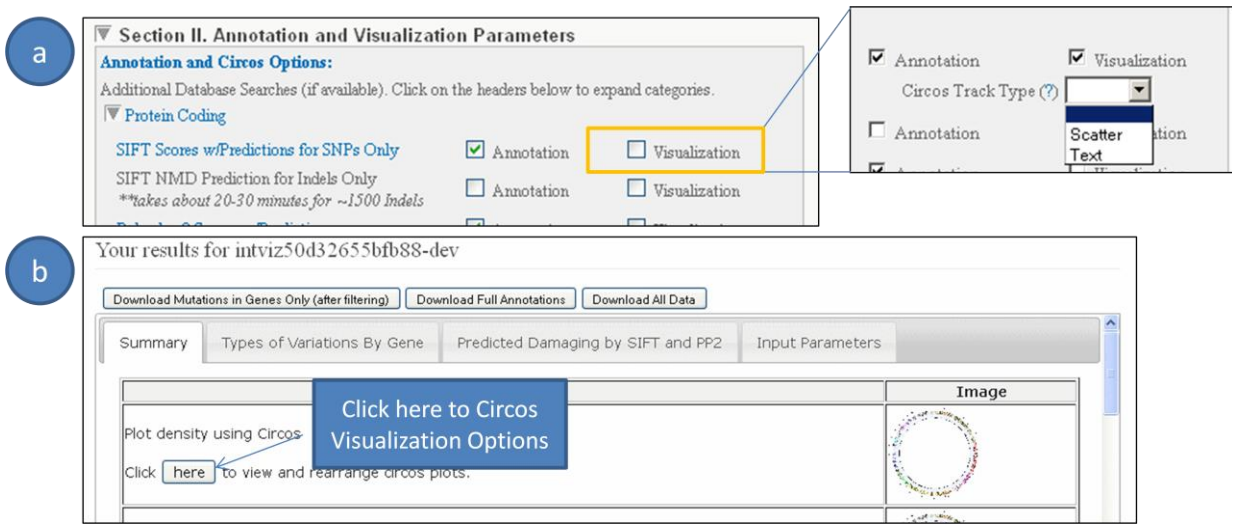
Figure 34. Input Parameters Tab



2) Circos Visualization: Viewing and rearranging plots

Based on user request, AVIA generates Circos Plots of the user's input variation data. A unique feature in AVIA is that users can rearrange multiple single plots generated by the AVIA pipeline into a multi-Circos plot without having to resubmit a request. Recall from Workflows Section of the Tutorial, there were two options for each database in the Annotation and

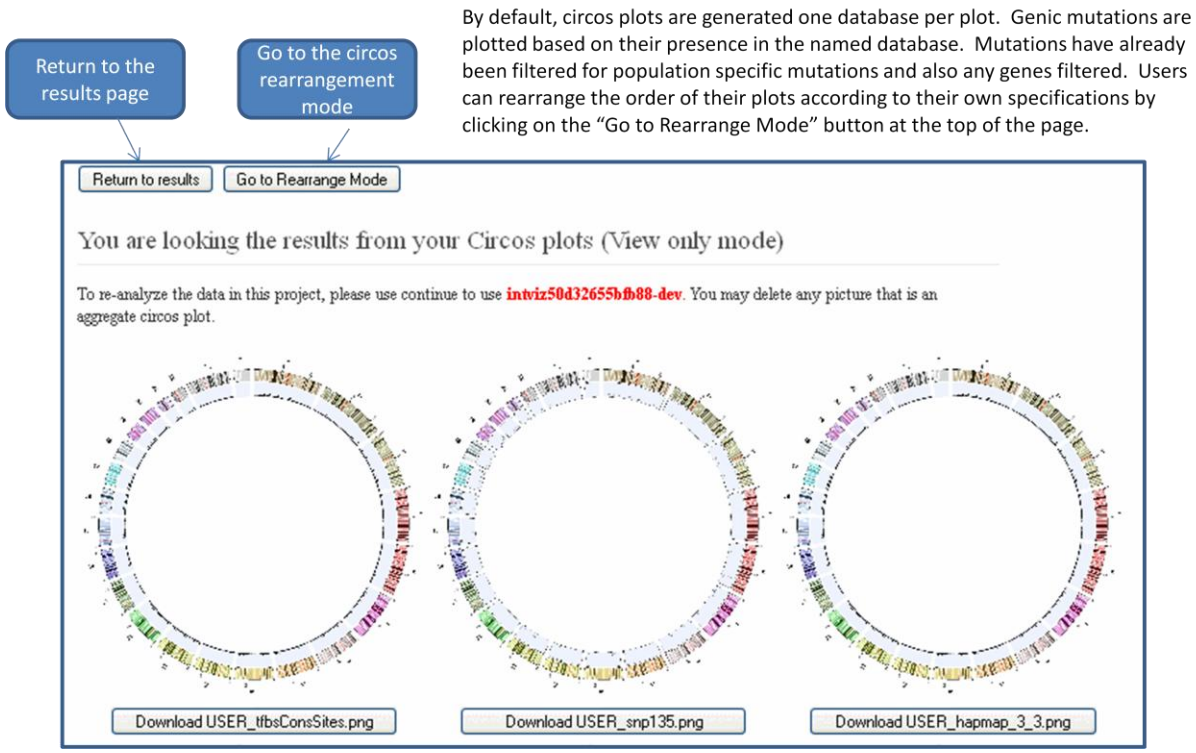
Figure 35. Circos Visualization Checkbox(es)



Visualizations Section; one for "Annotation" and one for "Visualization" as seen in Figure 35a.

Once the Visualization box was selected, an additional option to select the "Circos Track type" appeared, inset Figure 35a. If you select this option, you will be able to continue to the visualization portion described next. If you selected multiple databases for visualization, then you will have Circos images on your Results landing page to click on, as seen in Figure 35b. By clicking on any of the "Click here to view and rearrange Circos Plots" buttons on your results landing page, you will be redirected to the page with all of your Circos plots.

Figure 36. Circos Visualization Page

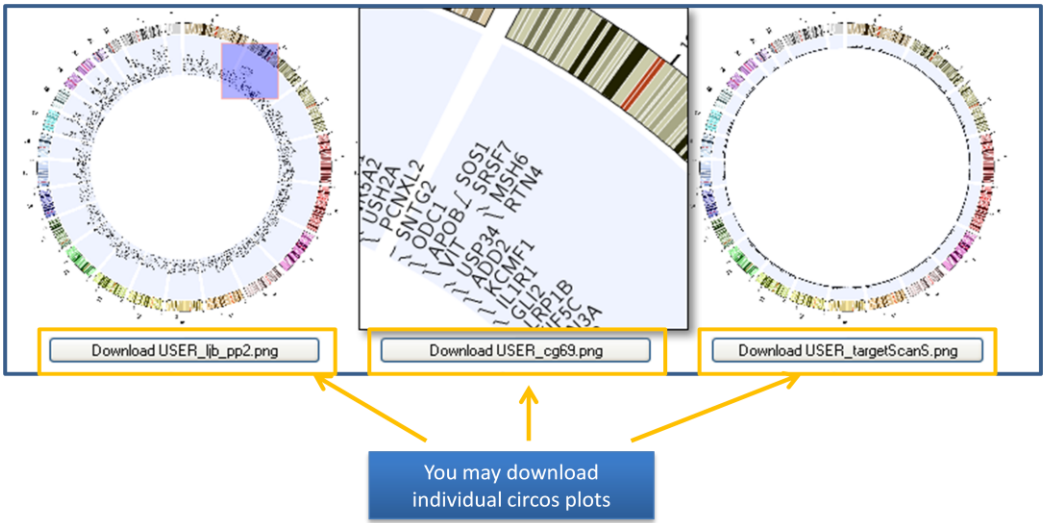


The following pages are Circos specific pages and may not be of interest if you only have one plot. Figure 36 (above) is the first Circos visualization page with your user data.

On the page shown in Figure 37, you can view all of your plots. You can zoom in and move around the Circos plots to take a closer look (Figure 37, inset). You also have the option to download individual plots by clicking on the download button below the Circos plots. (orange boxes in Figure 37). This page is strictly for viewing plots. In order to combine multiple plots for publication or general viewing, you can navigate to the "Rearrange Mode" on the top of the page.

Figure 37. Circos Visualization Page Zoom Option

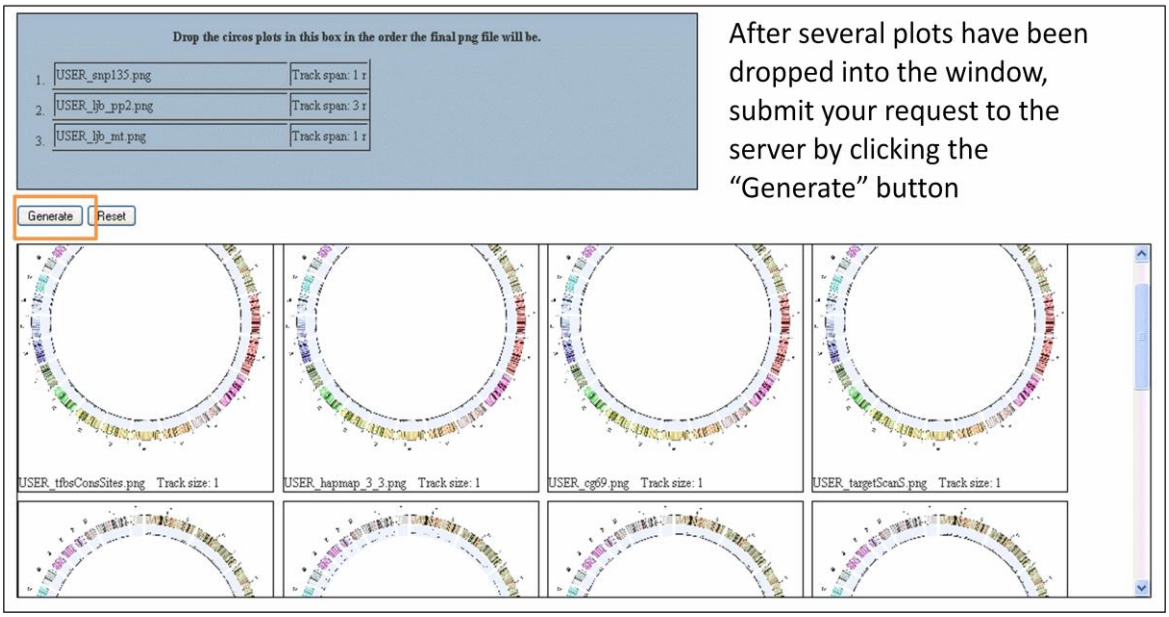
On this page, users may also zoom into their circos plots. Hovering over the plot will magnify the region in the light gray box.



By clicking on the "Rearrange Mode" on the top of your Circos page, you will be directed to the rearrangement mode. This is where you can make Circos plots with more than one ring.

Figure 38. Circos Rearrange Page.

Circos plots can be dragged and dropped into the grey window. Plots will disappear from the lower window when dropped in the grey window. The order in the window will determine the rings of the circos plot.



After several plots have been dropped into the window, submit your request to the server by clicking the "Generate" button

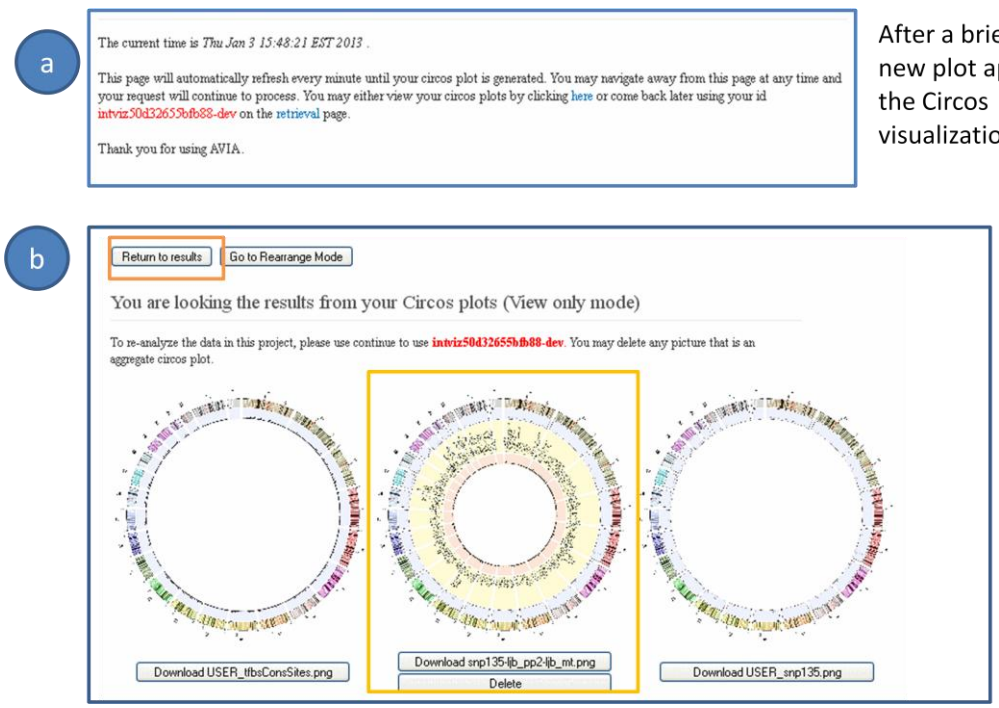
Only single plots with single rings will be displayed here. Each of the plots on the bottom

panel can be dragged to the light gray box. The name and track span will appear in the table in the gray box if it has been successfully added. Each row in the table represents a ring within the Circos Plots in the order that it appears in the table. In the example shown in Figure 38, we plotted the data separately using 3 databases: snp135, ljb_pp2 and ljb_mt. We dragged the individual Circos Plots to the gray box so that we will generate a new Circos plot with three rings representing each of the three databases added in the order added to the gray box. You may then click on the "Generate" button, shown in the orange box, to send to AVIA to redraw your picture.

After a brief wait (Figure 39a), a new Circos plot with your tracks inserted in the order requested as shown in Figure 39b, large yellow box. You may do this multiple times.

You can return to your results page to download all of your data by clicking on the "Return to results" (shown in the orange box in Figure 39b) button at the top left corner of the navigation pane. From that page, there are several buttons to choose which data you wish to download, including your newly created Circos plots.

Figure 39. Circos Processing and New Plot Page



After a brief wait, a new plot appears in the Circos visualization page.

3) Downloading Results Archive

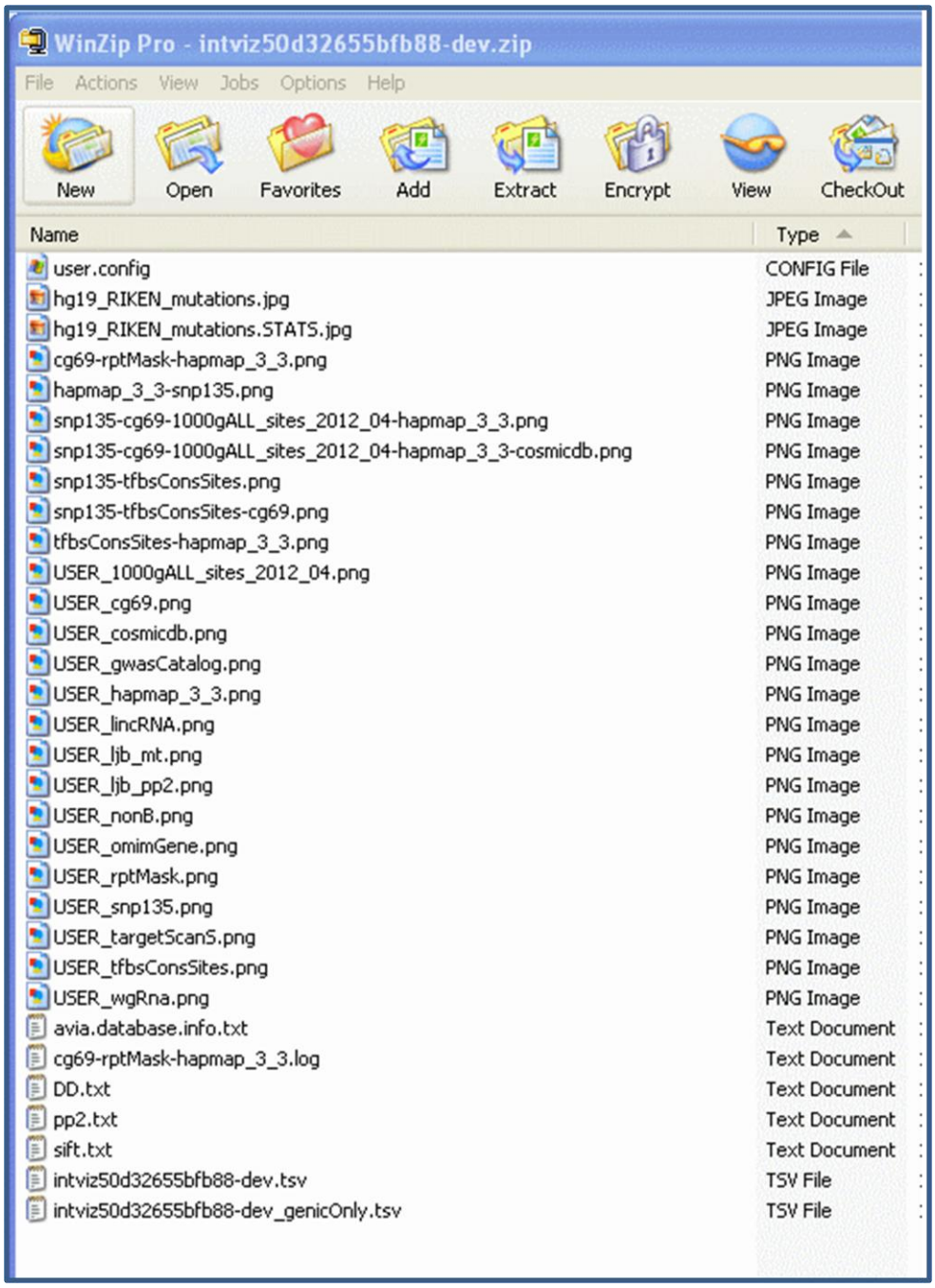
We have shown many places where you can download individual files. On the main results page, there is an option to "Download All Data" for your entire request. Below we describe in detail the contents included in the archive and how to view the data.

The contents of this zipped file can be viewed with any zip program such as WinZip (http://www.winzip.com/win/en/index.htm) or 7-Zip (http://www.7-zip.org/). Your archive may vary in number of files and different file names; however many of the files have standardized AVIA names. Filenames followed by asterisks (*) indicates that it is a name derived based on the user's original input filename. In the example shown in Figure 38, the input of our variants file was 'hg19_RIKEN_mutations.txt'. Some of the filenames may have derived from the user's original filename, which are in the light pink font. Other filenames are fixed or dependent on the database used.

- **user.config** : shows your inputs to AVIA, including your annotation and Circos options
- ***jpg files:** shows statistics in the user's variant data
    1. Venn diagram of damaging mutations according to sift and polyphen2 and snps found in dbSNP **(ex. "**hg19_RIKEN_mutations**.jpg")**
    2. Pie chart of exonic mutation types (ex. "hg19_RIKEN_mutations**.STATS.jpg**")
- ***png files :** show user's Circos plots using the name of the database plotted.
    1. Any files beginning with "USER" are the single Circos plots. e.g USER_cg69.png
    2. All others are the names of the multi-level plot and the names reflect the order in which the rings appear.
       eg. Cg69-rptMask-hapMap_3_3.png indicates a 3 ring Circos plot with cg69 mutations, rptMasker mutations and hapMap mutations plotted.
- **avia.database.info.txt :** file containing database information used for your input, including the build and other significant information.
- **DD.txt:** file that contains all mutations found damaging by both SIFT and Polyphen2.
- **pp2.txt:** file containing mutations that were damaging by Polyphen2.
- **sift.txt:** file containing mutations that were found damaging by SIFT.
- ***tsv files:** tab delimited files containing all your annotation for your input file using your unique AVIA identifier (*_genicOnly.tsv contains only genic mutations)

An example archive is shown in Figure 40. Your archive may more or less files than this depending on your input selections. In the user.config file, it will show which version of AVIA that was used in analysis.

Figure 40. AVIA Archive Contents



B) Viewing Results on Local Computer

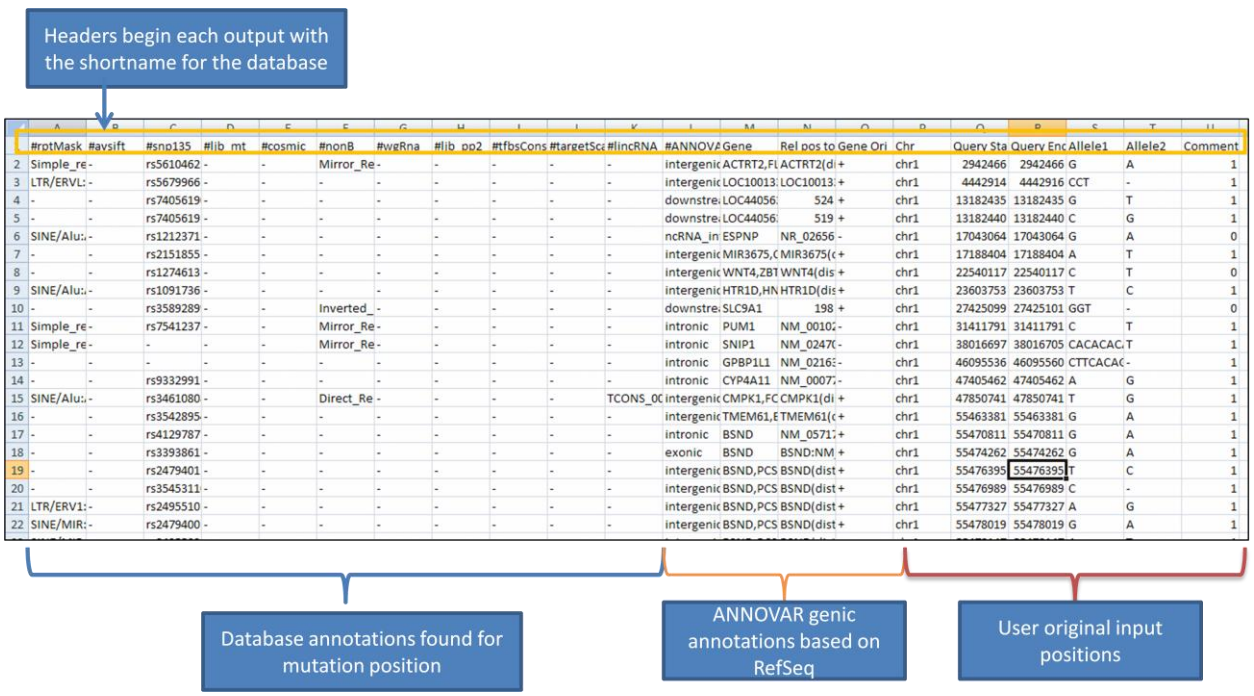
1) AVIA Output Annotation File (tsv)

The main output of AVIA is the annotation in a tab separated file which can be viewed in any text editor. In Figure 41, we show an example using Microsoft Excel®. The first line is always the

header to describe what is in the column. Each annotation database is shown in the columns preceding genic annotation and user data. Each header is a brief descriptor of the contents of the column. Each line corresponds to variation data from the user's original input, each of the columns refers to any annotation associated with the database for a specific mutation. In Figure 41, we show that annotations were requested for rptMask, avsift, snp135, ljb_mt, cosmic, nonB, wgRna, ljb_pp2, tfbsConsSites, targetScan, and lincRNA. Whenever possible, we try to replace the database names with more meaningful descriptors. If you do not know to which databases the column belongs, you can refer to our "Databases">"View Databases" option under the User Navigation Menu match the "ANNOVAR Abbrv Name" to these headers.

DD.txt, pp2.txt, sift.txt, and *genicOnly.tsv are fixed filenames and are subsets of this file and are best viewed using Excel®. Other text files are best viewed using text editors such as Notepad ® ,Wordpad ® or Microsoft Word ®.

Figure 41. AVIA Annotation Output File

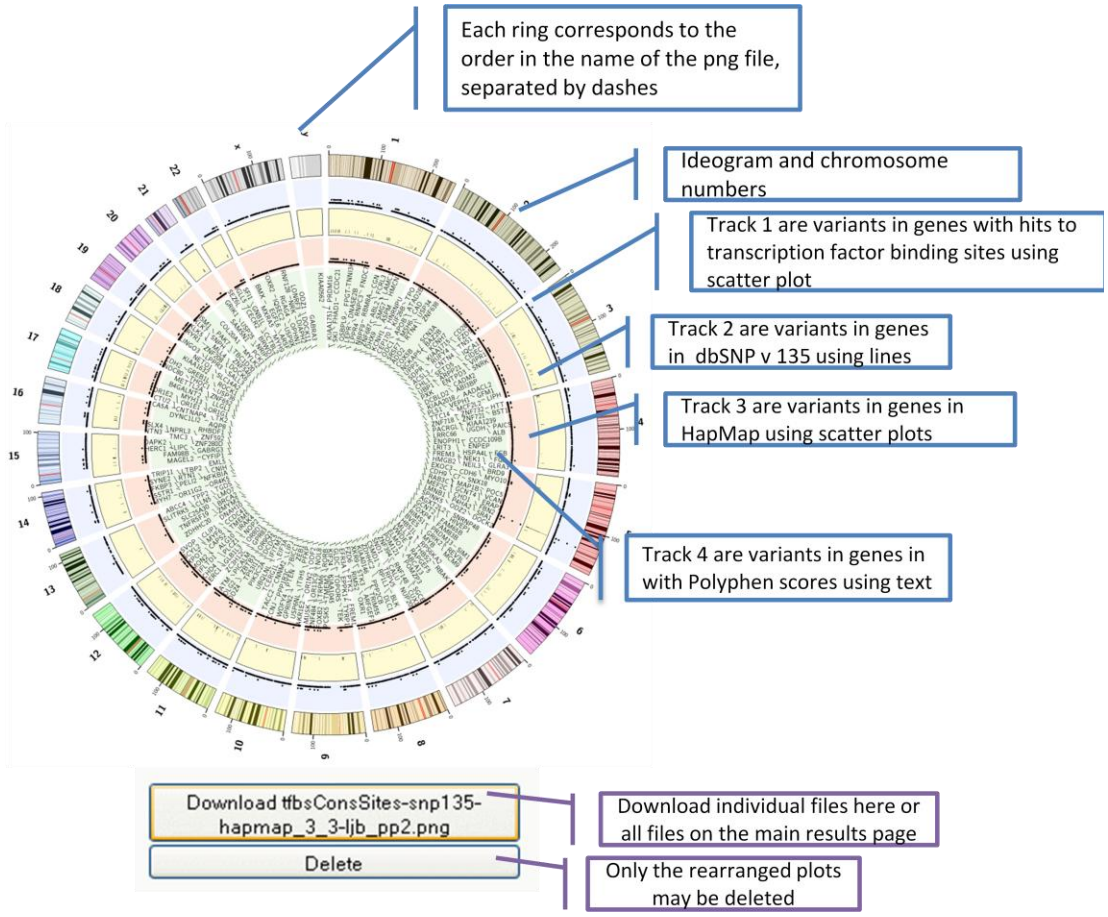


	#rptMask	#avsift	#snp135	#lib_mt	#cosmic	#nonB	#wgRna	#lib_pp2	#tfbsCons	#targetSca	#lincRNA	#ANNOVA	Gene	Rel pos to	Gene Ori	Chr	Query Sta	Query End	Allele1	Allele2	Comment
2	Simple_re	-	rs5610462	-	-	Mirror_Re	-	-	-	-	-	intergenic	ACTRT2,FL	ACTRT2(di	+	chr1	2942466	2942466	G	A	1
3	LTR/ERVL:	-	rs5679966	-	-	-	-	-	-	-	-	intergenic	LOC10013	LOC10013	+	chr1	4442914	4442916	CCT	-	1
4	-	-	rs7405619	-	-	-	-	-	-	-	-	downstre	LOC44056	524	+	chr1	13182435	13182435	G	T	1
5	-	-	rs7405619	-	-	-	-	-	-	-	-	downstre	LOC44056	519	+	chr1	13182440	13182440	C	G	1
6	SINE/Alu:	-	rs1212371	-	-	-	-	-	-	-	-	ncRNA_in	ESPNP	NR_02656	-	chr1	17043064	17043064	G	A	0
7	-	-	rs2151855	-	-	-	-	-	-	-	-	intergenic	MIR3675,C	MIR3675(c	+	chr1	17188404	17188404	A	T	1
8	-	-	rs1274613	-	-	-	-	-	-	-	-	intergenic	WNT4,ZBT	WNT4(dis	+	chr1	22540117	22540117	C	T	0
9	SINE/Alu:	-	rs1091736	-	-	-	-	-	-	-	-	intergenic	HTR1D,HN	HTR1D(dis	+	chr1	23603753	23603753	T	C	1
10	-	-	rs3589289	-	-	Inverted_	-	-	-	-	-	downstre	SLC9A1	198	+	chr1	27425099	27425101	GGT	-	0
11	Simple_re	-	rs7541237	-	-	Mirror_Re	-	-	-	-	-	intronic	PUM1	NM_0010	-	chr1	31411791	31411791	C	T	1
12	Simple_re	-	-	-	-	Mirror_Re	-	-	-	-	-	intronic	SNIP1	NM_0247	-	chr1	38016697	38016705	CACACAC	T	1
13	-	-	-	-	-	-	-	-	-	-	-	intronic	GPBP1L1	NM_0216	-	chr1	46095536	46095560	CTTCACA	-	1
14	-	-	rs9332991	-	-	-	-	-	-	-	-	intronic	CYP4A11	NM_0007	-	chr1	47405462	47405462	A	G	1
15	SINE/Alu:	-	rs3461080	-	-	Direct_Re	-	-	-	-	TCONS_0	intergenic	CMPK1,FC	CMPK1(di	+	chr1	47850741	47850741	T	G	1
16	-	-	rs3542895	-	-	-	-	-	-	-	-	intergenic	TMEM61,E	TMEM61(c	+	chr1	55463381	55463381	G	A	1
17	-	-	rs4129787	-	-	-	-	-	-	-	-	intronic	BSND	NM_0571	+	chr1	55470811	55470811	G	A	1
18	-	-	rs3393861	-	-	-	-	-	-	-	-	exonic	BSND	BSND:NM	+	chr1	55474262	55474262	G	A	1
19	-	-	rs2479401	-	-	-	-	-	-	-	-	intergenic	BSND,PCS	BSND(dist	+	chr1	55476395	55476395	T	C	1
20	-	-	rs3545311	-	-	-	-	-	-	-	-	intergenic	BSND,PCS	BSND(dist	+	chr1	55476989	55476989	C	-	1
21	LTR/ERV1:	-	rs2495510	-	-	-	-	-	-	-	-	intergenic	BSND,PCS	BSND(dist	+	chr1	55477327	55477327	A	G	1
22	SINE/MIR:	-	rs2479400	-	-	-	-	-	-	-	-	intergenic	BSND,PCS	BSND(dist	+	chr1	55478019	55478019	G	A	1

2) How to Read the Circos Plot Names

Below (Figure 42) is an illustration of how to interpret the Circos rings using the filename. In order to name figures uniquely and condense names, we had to abbreviate the names of the databases. Please refer to the "Databases">"View Database" in the User Navigation Panel along the top of any AVIA page.

Figure 42. How to Read a AVIA-Circos Rearranged Plot



3) Viewing graphics files

Each computer is different, but all of the graphics files (Venn Diagrams, Circos Plots) that end in ".png" or ".jpg" can be viewed with your computer's default image viewer. If you cannot open them, refer to your computer's user manual to find the appropriate program to view these types of files.

VIII) Reusable Configuration File

As you become more accustomed to the AVIA pipeline, you can set up a configuration file in which the minimal databases and features will be applied to each of the user's submitted runs, without having to click each of the desired database and features. Users will still need to fill out the "Input Data" section

each time they upload, but based on the same email address used for the configuration set up, all of the queries and features will be applied with the new data file. This applies only to the "Annotation and Visualization" section of the web pages. On the individual submissions page, users may always add databases and features to the output. Users can also change their configuration at any time by following the directions on the page and confirm each submission by email. We currently do not accept batch loading of data files at this time.

To set up a configurations file, navigate to "User Resources" from the top navigation pane, and select "Set up a configurations file" as indicated by Figure 43a yellow arrow and box. You have two choices for the Workflow: Feature Annotation/Visualization and Cascade Filtering. Although these workflows are similar and have the same features, we found that not all users will want to filter their dataset each time so we have separated these into two distinct workflows shown in Figure 43b. The Cascade filtering workflow includes the Feature Annotation and Visualization. When you have completed the form, you will receive an email confirmation and once this link is activated by following the provided link, your configurations will be set up for every subsequent submission for the same workflow. If you wish to change your configurations, you may go to the same link, and change your information there. It will prompt you to delete your previous submission and will email you another confirmation link to activate your configurations file.

Figure 43. Set up a Configurations File

IX) References

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