USER GUIDE

Alamut Visual Plus

SOPHiA GENETICS™

SUMMARY INFORMATION

Product Name	Alamut Visual Plus
Product Type	Analytical/Visualization software
Product Family	Software
Reference (Product Code)	LAVP
Product Version	1.1
Document ID	ID-60101-22
Document Version	1.1
Revision Date	22.04.21

Please read this User Manual thoroughly before using this product.

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REF LAVP

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REVISION HISTORY

DOCUMENT ID/VERSION NUMBER	DATE	DESCRIPTION OF CHANGE
ID-60101-22 v1.1	23.04.2021	Title page, company logo changed. Header and Footer modified. Disclaimer page shifted to page 2. Page 8: Section 5.1, Internet Connection - IP addresses modified. Page 44-45: Section 10.7.2 Importing Variants - Section elaborated. Page 54-61: Section 10.8.3 to 10.9.4 - New sections added. Last page added.
ID-60101-22v1.0	04.03.2021	Initial Release

Table of Contents

1. Product Description

Alamut® Visual Plus is a comprehensive genome browser compliant with HGVS nomenclature and powered by multiple genomic sources and prediction tools displayed in an interactive interface to ease variant interpretation.
Alamut® Visual Plus is an upgrade of Alamut® Visual.

2. Intended Use/Purpose

2.1. Intended Use

Alamut® Visual Plus is a Research Use Only software that eases daily genetic analysis. Alamut Visual Plus allows the visualisation of variants, transcripts, genomic sequences and genomic data to simplify variant interpretation and pathogenicity assessment.

2.2. Intended User Profile

The application is intended to be used by trained medical professionals (clinicians, researchers, research technicians, …) working in the field of Genomics.

2.3. Intended Use Environment

The place of use is determined to be in a hospital, labs or clinical setting, on a regular computer.

3. General Statement of the Test Principle(s) / Procedure

Alamut® Visual Plus general procedure is as follows:

- Display of a genomic region
- Access to multiple public genomic sources and call prediction tools
- Search for specific genomic data (genes, transcripts, variants)
- Manual creation of genomic variants
- Import and export of private variant annotations
- Reporting of variant data

4. Product Components

The product is only composed of the Alamut® Visual Plus software.

Alamut® Visual Plus is installed with a floating license. Floating licenses can be installed on multiple computers, with a limited number of concurrent users and are managed via a web page (extranet).
Alamut® Visual Plus extranet page: http://extranet.interactive-biosoftware.com.

5. Specifications and Installation

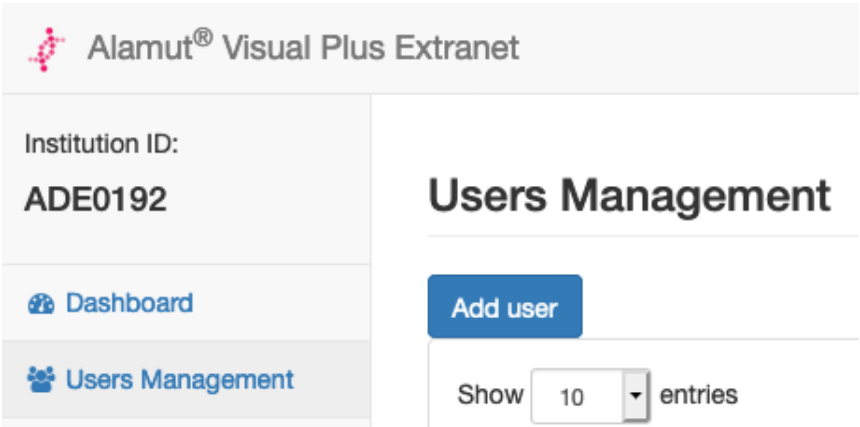
5.1. Specifications

Alamut® Visual Plus is designed to be used on computers with a keyboard and mouse.
Alamut Visual Plus is meant to be installed on computers meeting the following technical specifications:

System Component	Minimum Requirement
Operating system	Microsoft Windows 10, 64-bit version The program is available as an installer program (.exe) or self-extractable archive (.exe) or compressed file (.zip) Mac OS X- starting from Mac OS 10.14 (Mojave)
Internet Connection	Connection to the following IP addresses are required: • 62.210.147.42 • 212.83.147.70 The software handles connections through HTTP/HTTPS on port 80 and 443, optionally through a proxy server.
Hardware Requirements	Computer: 1.5GHz+ - 8GB RAM - 500MB free disk space. Display screen resolution: 1024x768 pixels The software program does not alter system directories or the registry. Write permissions are required on the software directory to ensure continued functioning of the application and to save user parameters.

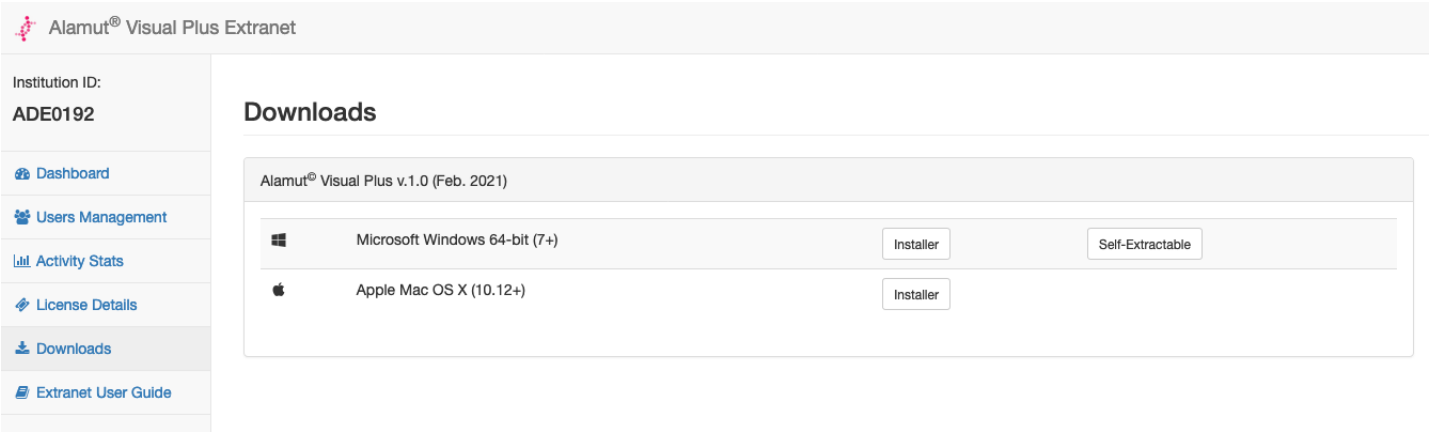
5.2. Installation

Once the Administrator's credentials have been created, the Administrator must log-on to the Alamut® Visual Plus extranet page and create a new user account through the "Add user" button.



The new user will receive the Institution ID, User Name and Password via e-mail.
In order to install the program, the new user has to open the Alamut® Visual Plus extranet page and sign in with the Institution ID, User Name and Password provided.

Alamut® Visual Plus binaries can be downloaded from the “Downloads” section.



Installation instructions depend on the computer’s operating system.

Microsoft Windows

Download the Alamut® Visual Plus Installer or Self-Extractable executable (.exe) from our extranet page: http://extranet.interactive-biosoftware.com.

- If you have downloaded the Installer: execute it and choose an installation folder where you have write permissions
- If you have downloaded the Self-Extractable executable (.exe) file, simply double-click on the installer to launch the installation program and follow the instructions presented to you. Select a folder where you have write permissions.

After you have finished installing the program you should remove the files used for the installation: the Installer file or the Self-Extractable executable (.exe) file.

Mac OS X

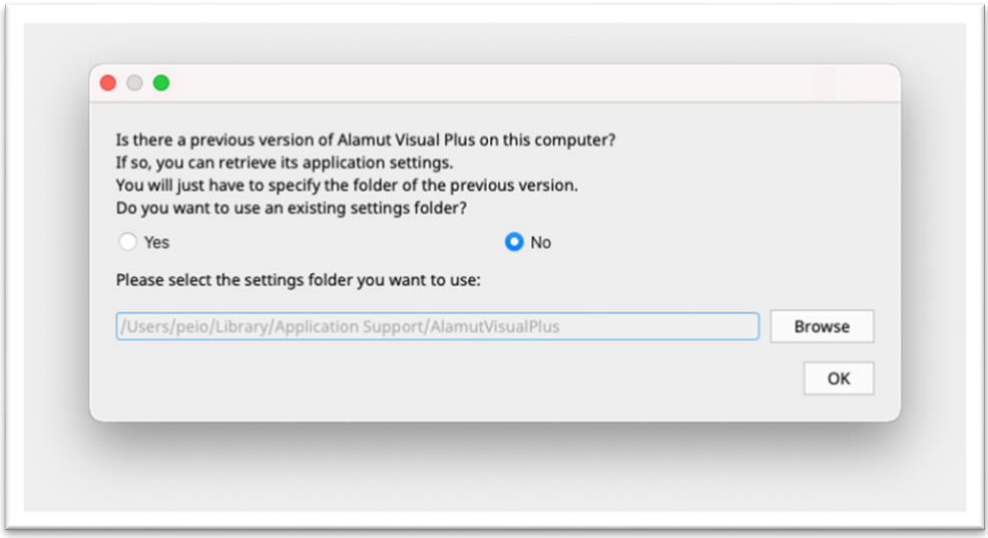
Download the Alamut® Visual Plus (**.dmg**) file from the extranet page: http://extranet.interactive-biosoftware.com.

- double click the DMG to make its content available
- drag the application from the **.dmg** window into /Applications to install (may need an administrator password)
- wait for the copy process to finish
- eject the **.dmg** (by clicking the eject button in the Sidebar)
- delete the **.dmg** from Downloads

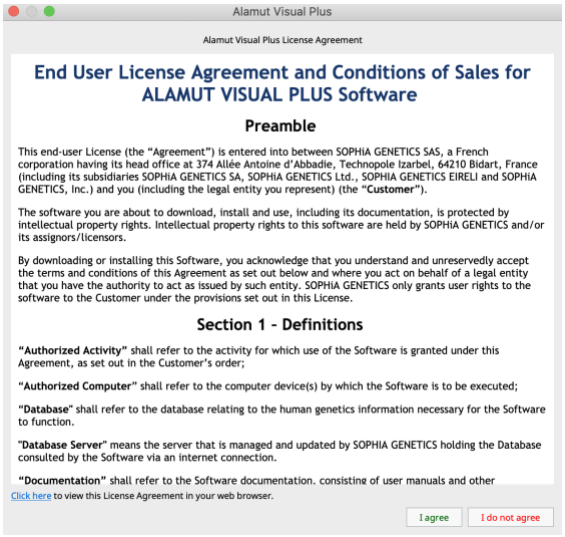
5.3. Post installation

Once Alamut® Visual Plus is installed, you must set it up. Launch Alamut® Visual Plus and:

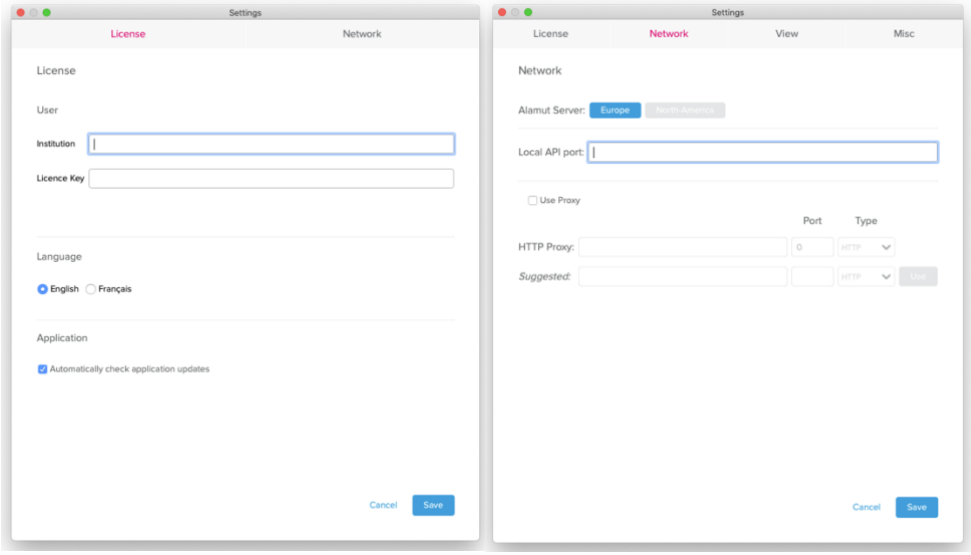
1. Define the application's data folder, where Alamut® Visual Plus will store its settings. You may decide to reuse an existing folder created by an older version of Alamut® Visual Plus: in this case, saved settings will be restored.



2. Accept the End User License Agreement.

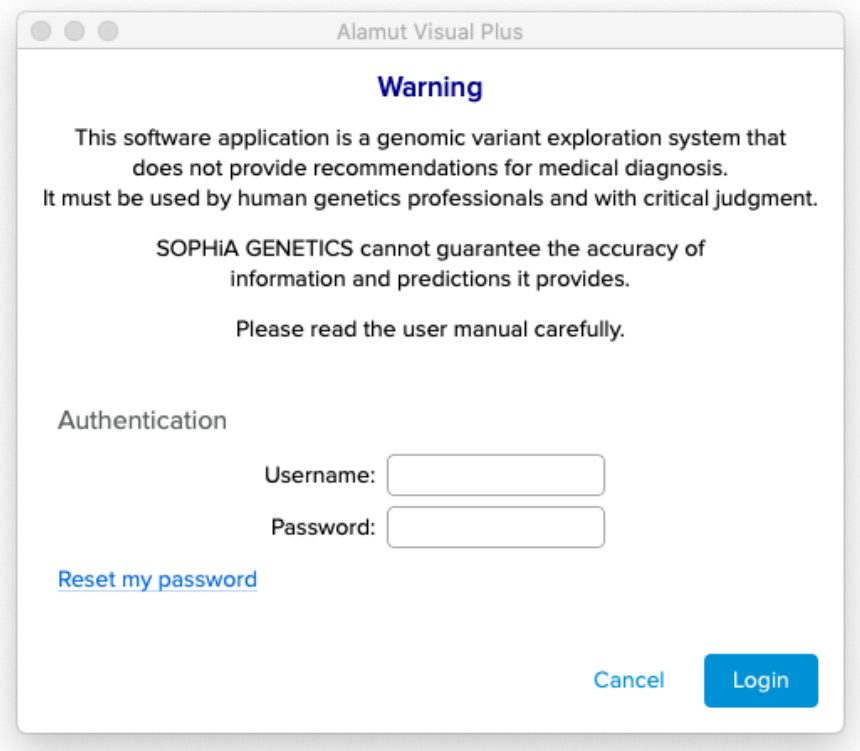


3. Provide
    a. Your institution code and your license key
    b. If any, information relating to your proxy server



These 3 steps have to be followed at the **first launch only**.

Once completed, users will only have to provide their username and password to connect to the application. The password will be renewed by the user every 90 days.



6. Storage and Handling

6.1. Storage

Not applicable.

6.2. Handling

Not applicable.

7. Warnings, Limitations and Precautions

For Research Use Only. Not for use in diagnostic procedures.
Alamut® Visual Plus does not provide recommendations for medical diagnosis. It must be used by human genetics professionals and with critical judgment. SOPHiA GENETICS does not guarantee the accuracy of information and predictions it provides.

8. Residual Risks

No residual risk has been identified as part of Alamut Visual Plus risk assessment.

9. Equipment and Materials Required, Not Provided

The user needs to provide a computer, a keyboard, a mouse and an internet connection according to section 5 of this user guide.

10. User Manual

10.1. Support

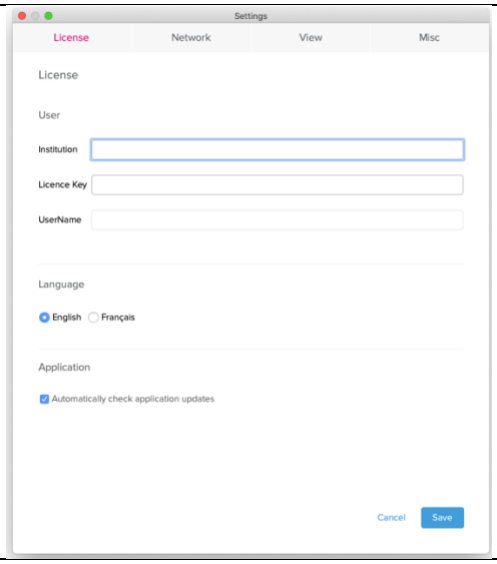
To get support for Alamut® Visual Plus, you can contact us by:

- clicking on Help on the Alamut® Visual Plus menu: Contact Support
- sending directly an email to the Technical Support team using the following email address: support@sophiagenetics.com

10.2. Getting started: settings

The Application Settings window includes the following tabs: License, Network, View, Misc. (Miscellaneous)

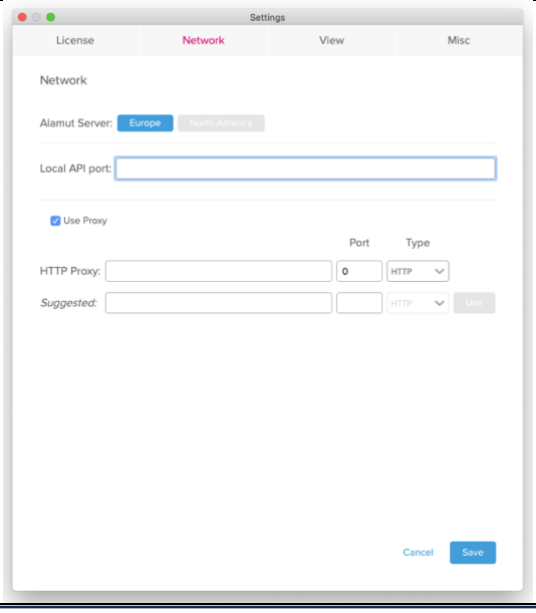
License

Institution: the code of the institution that purchased the license **License Key:** the license key of the institution **User Name:** the name of the connected user **Language:** Select your preferred language (English or French). **Application:** Tick the box to automatically check for updates.	

Click Save before leaving the tab. These details will be saved for future log-ins.

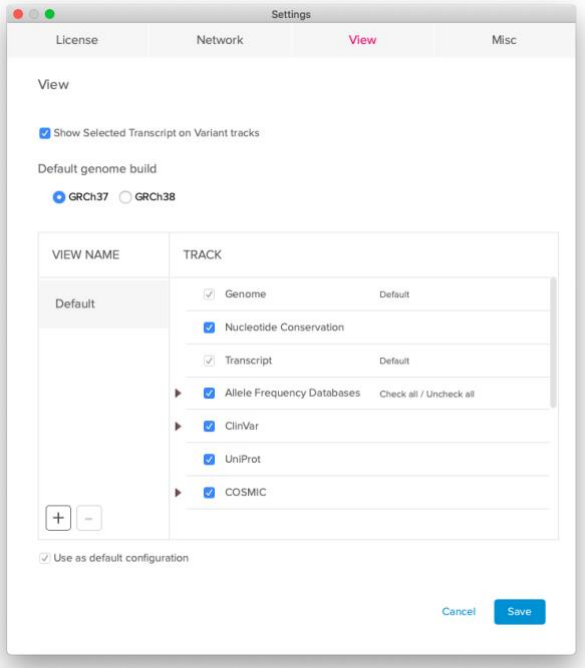
Network

To connect via proxy complete the necessary information in the Network tab (this may require the input of your IT administrator).

Use Proxy: To use a proxy server, tick the Use Proxy box and provide the required information to connect to it.	

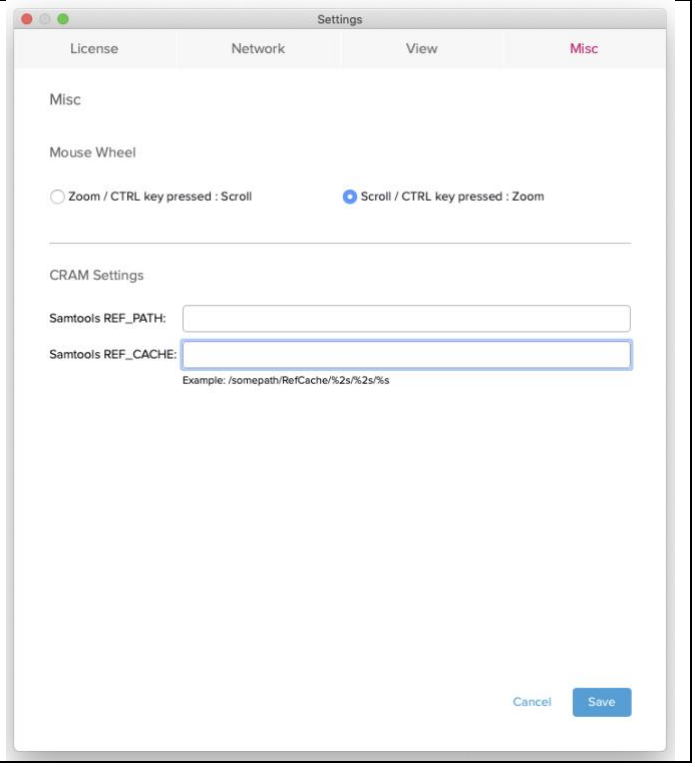
View

The View tab in the Application Settings window is where you can modify the configuration of the tracks (displayed tracks and their positions) and define which filters apply to each track.

Show Selected Transcript on Variant Tracks: select to view the selected transcript on the tracks. **Default Genome Build (GRCh37 or GRCh38):** select which genome build you would like to use by default. **Configuration:** it allows you to create a customizable view by adding or deleting the tracks that you want to see or hide. You may also reorder tracks via drag&drop. By clicking in 'New' you can create a new view configuration. In the *Default* configuration, all tracks are displayed.	

Note: configuration of tracks only applies to views where a transcript is selected.

Miscellaneous

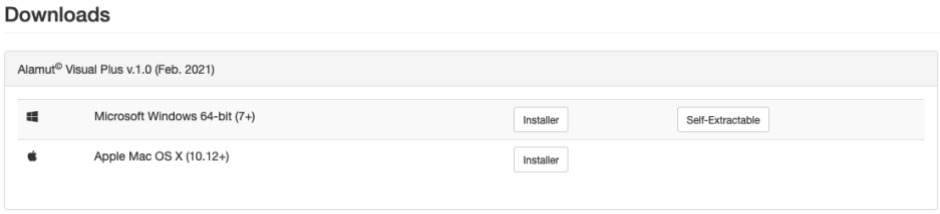
Mouse Wheel: two mouse scroll options are available to define the behaviour of the application when using only the mouse wheel or the mouse wheel in combination with the CTRL key. **CRAM Settings**: CRAM handling in Alamut is based on Samtools. Samtools needs the reference genome sequence in order to decode a CRAM file. Samtools can use either the REF_PATH or REF_CACHE environment variables to find reference sequences. (see below)	

Samtools needs the reference genome sequence in order to decode a CRAM file. It uses the MD5 sum of each reference sequence as the key to link a CRAM file to the reference genome used to generate it (see also the Samtools man page). You will need to provide the path to MD5 reference sequences in the REF_PATH or REF_CACHE field, unless you use CRAM files with embedded reference sequences.
For your convenience we have prepared a package of reference MD5 files for GRCh37 and GRCh38 primary sequences. It is available at:
http://downloads.interactive-biosoftware.com/CRAM/RefCache.tgz

For instance, if you uncompress this file to the D:\SAM folder under Windows, the REF_CACHE should be: D:\SAM\RefCache\%2s\%2s\%s

10.3. Manual update

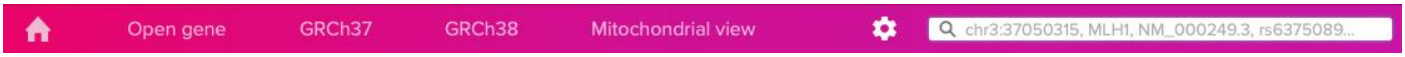
If you wish to update the application manually please follow these steps. Connect to our extranet page (http://extranet.interactive-biosoftware.com), select the downloads section and if available download the latest version.



- Or click on the “Update” button in the left part of the Alamut® Visual Plus homepage

10.4. Dashboard

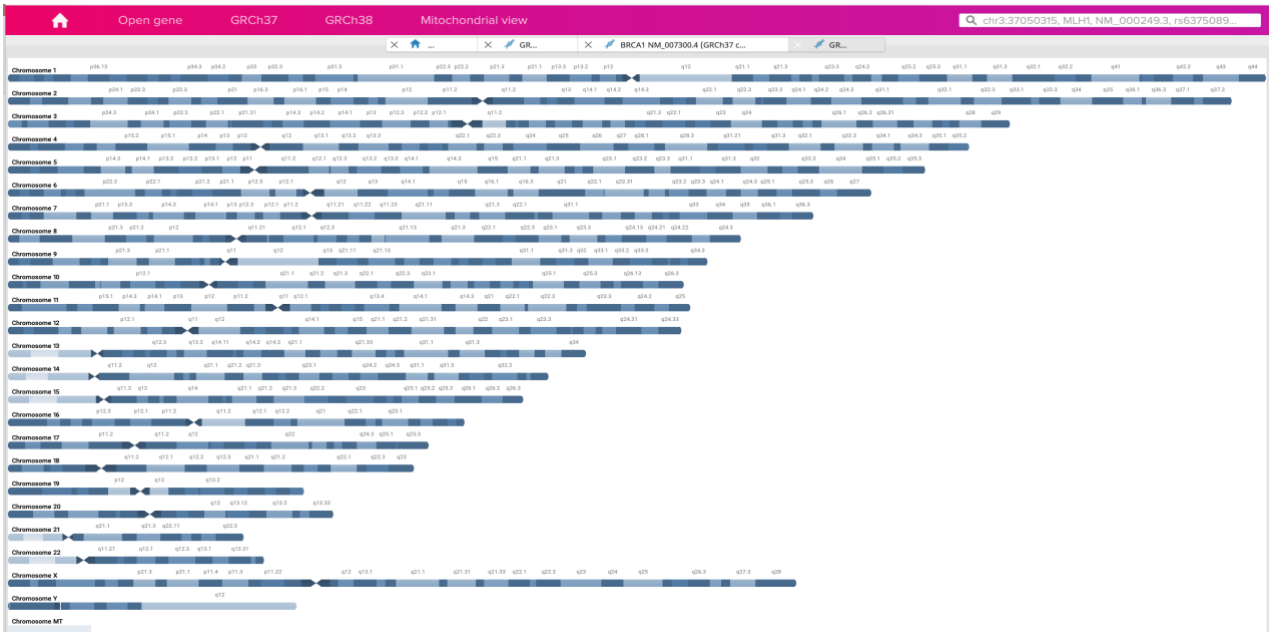
From the homepage you can open a gene, a genome assembly (GRCh37, GRCh38 or the mitochondrial genome) or directly perform a search (extended access feature).



From the Open Gene menu, the user can type a gene symbol and open it in Alamut® Visual Plus. He may also use shortcuts. These shortcuts are totally configurable.

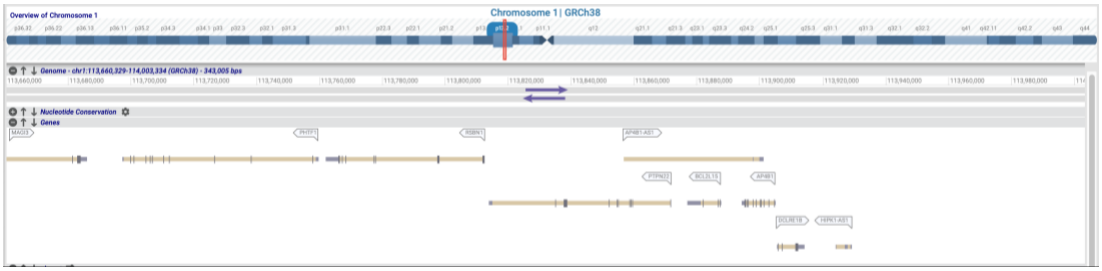
10.4.1. Genomic Views (GRCh37, GRCh38 and mitochondrial view)

The Genomic Views allow you to visualize all chromosomes including the mitochondria. You can visualize intergenic genomic regions, regulatory regions and structural variants.



You can select the chromosome you wish to study, by double clicking on it. The overview of the selected chromosome will open. You can select a specific chromosomic region and zoom in to

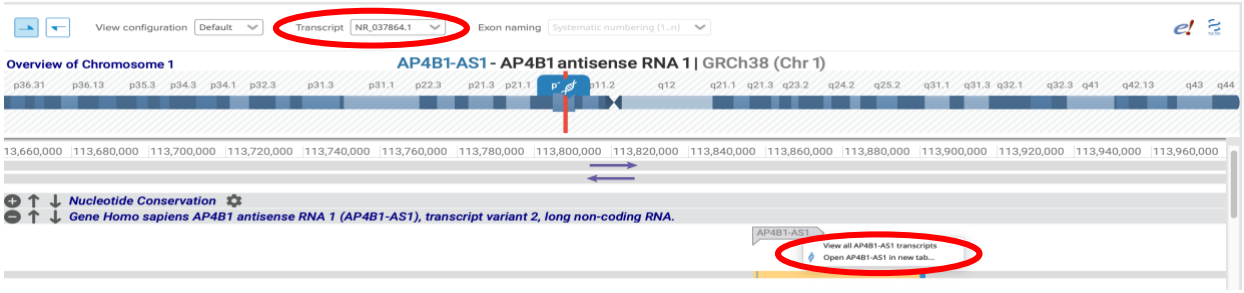
view the genes available in the selected band. Genes are displayed with named tags along the strand.



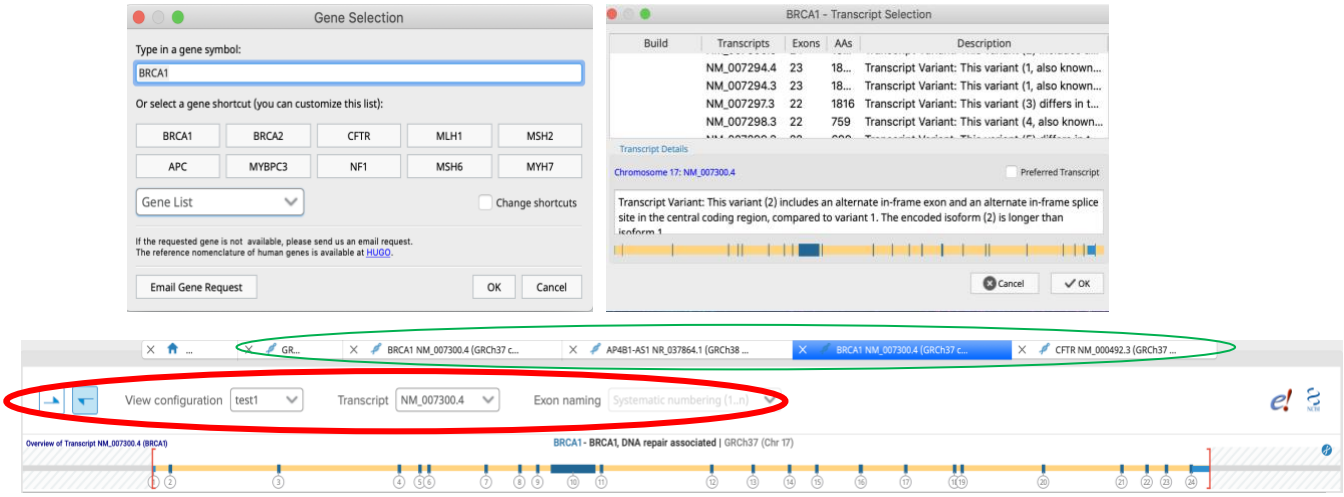
10.4.2. Overview of transcript

You can have access to the transcript view from:

1. the genomic views, by clicking on your gene of interest. You can display all transcripts related to one gene. The name of the transcript will be displayed in the transcript box.



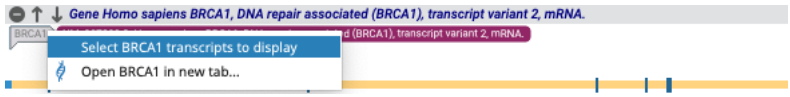
2. the ‘Open gene’ button in the homepage where you can select your gene of interest and then your transcript. The transcript of your selected gene is displayed in the overview, with the exons in blue and introns in yellow.



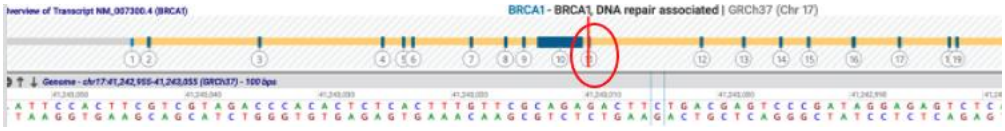
Notes

- The reading direction of the gene (i.e the DNA strand) can be changed by using the forward direction and reverse direction icons on the toolbar.
- The transcript and exon naming (if both Systematic and Custom naming are available for this transcript) can be modified interactively.
- The view configuration can be modified. You can use different View configurations in different tabs.
- Multiple tabs can be opened showing different genes/transcripts.
- It is also possible to display all available transcripts simultaneously.

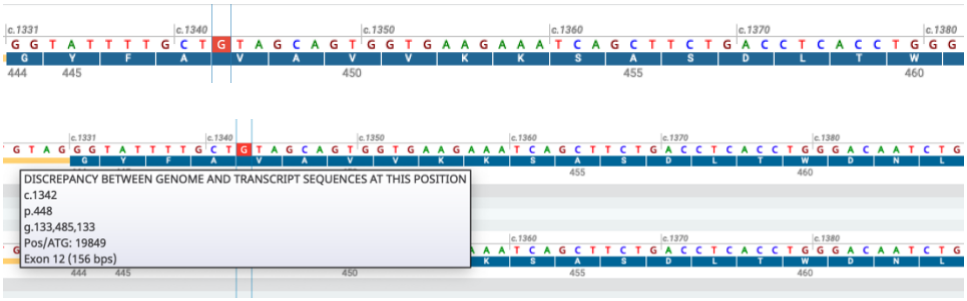
Several transcripts can be shown in the interface by exploring options in the transcript track by right clicking on the gene name.



Click on an exon number in the gene overview to zoom in directly and see the nucleotide sequence.



Note: Mismatches between transcript and reference genome sequences can be found. Transcript nucleotides are highlighted in **red** where they differ from the reference genome sequence and the tooltip gives a warning message.



10.4.3. Search bar (Extended Access Feature)

Alamut® Visual Plus provides a search bar allowing to access:

Standard Genetic References

Reference	Example
Official HGNC gene symbol	MLH1
HGNC Id	HGNC:7127
cDNA RefSeq Id	NM_000249.3

Ensembl Transcript Id (mapped to a NCBI RefSeq)	ENST00000231790
LRG Id	LRG_1
Protein RefSeq Id	NP_000240.1
UniProt Id	P40692
Reference SNP Id	rs63750891
OMIM Id	OMIM:120436

Genomic, cDNA and protein positions

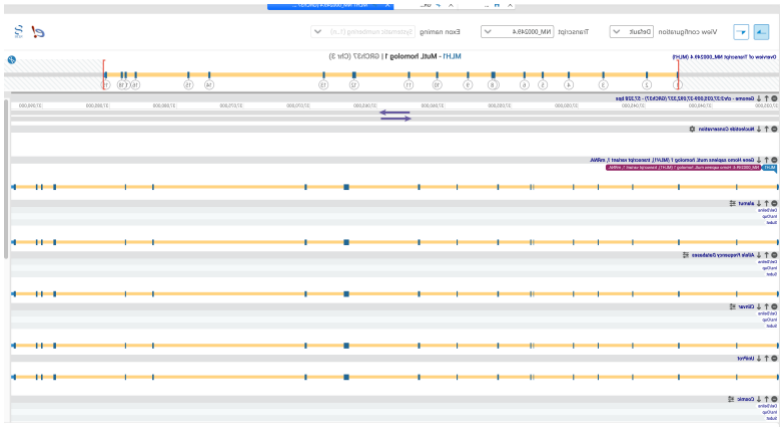
Reference	Example
Standard gDNA query	chr3:g.37050315
Short gDNA query	3:37050315
Interval gDNA query	3:36000000-38000000
gDNA query inside current gene	g.37050315
gDNA query with assembly	chr3(GRCh37):g.37059038
cDNA position query	NM_000249.3:c.464
Short cDNA position query	NM_000249.3:464
cDNA position query on Ensembl Trancript Id (mapped to a NCBI RefSeq	ENST00000231790:c.464
NM_000249.3:p.155	NM_000249.3:p.155
Protein substitution	NM_000249.3:p.Leu155Arg

Genomic and cDNA variants

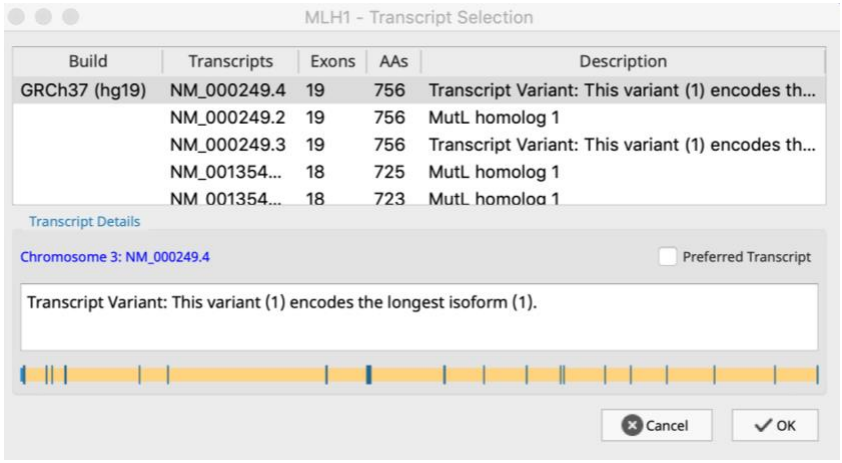
Reference	Example
Query with genomic variation	chr3:g.37050315T>G
Short query with genomic variation	3:37050315T>G
Use given assembly	Chr3(GRCh37):g.37067317G>A
cDNA variant	NM_000249.3:c.464T>G
cDNA variant on Ensembl Trancript Id (mapped to a NCBI RefSeq)	ENST00000231790:c.464T>G

Depending on the query type, Alamut® Visual Plus may:

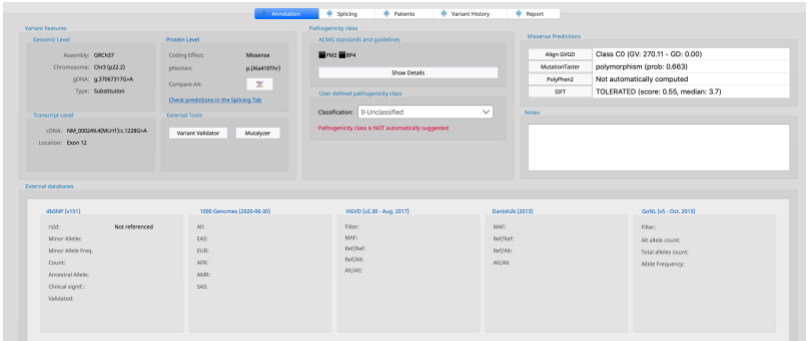
a. Open a gene and one of its transcripts



b. Open a transcript selection dialog



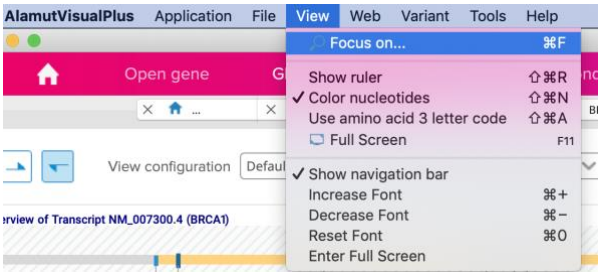
c. Open the variant panel



d. Directly focus to a genomic location

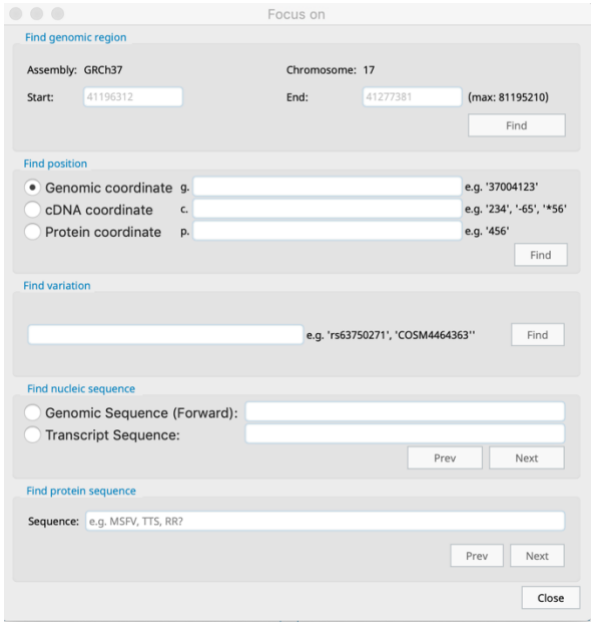
10.4.4. “Focus on” feature

Another feature ‘Focus on’ allows you to search for genomic coordinates and more. This feature is available from the “**View > Focus on**” menu.



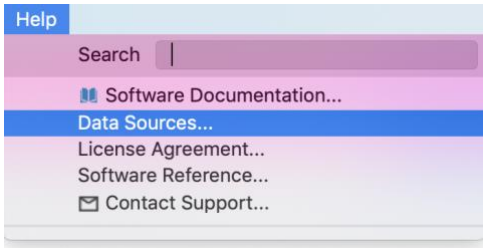
This functionality allows the user to easily and quickly find a:

- genomic region
- genomic position
- cDNA position
- protein position
- variation
- nucleic sequence
- protein sequence



10.5. Data sources

To view the up-to-date list of data sources integrated into Alamut® Visual Plus, go to **"Help > Data Sources"**. Database versions of each source is also available here.

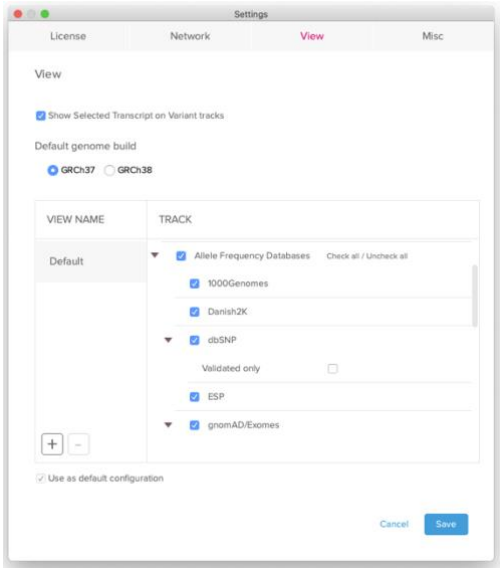


By default, all data sources are selected and displayed. You can enable or disable manually these data sources in each view configuration in **"Settings > View"**.

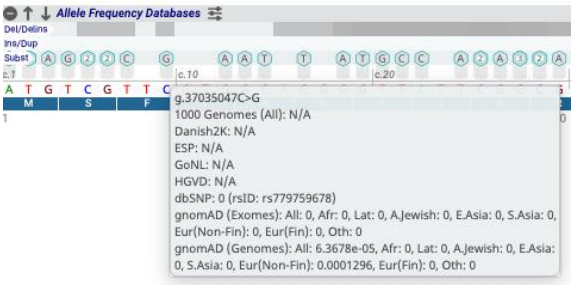
10.5.1. Population frequencies databases

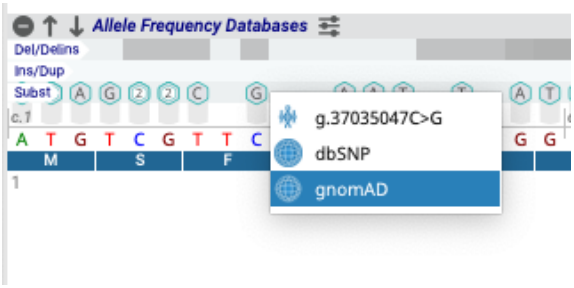
Alamut® Visual provides a convenient access to several databases of known variants.
Population frequencies data is displayed in one track: 'Allele Frequency Databases'.

You can configure your track by clicking on the 'Options' button. You can select or deselect data sources and choose filters:

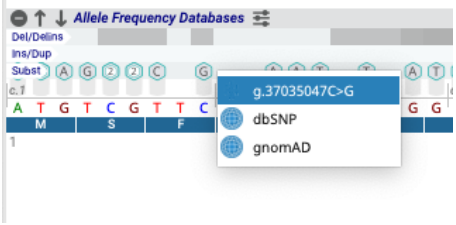


Hovering one variant will show information from different databases when available. Right clicking on a variant opens the official website of the selected source.

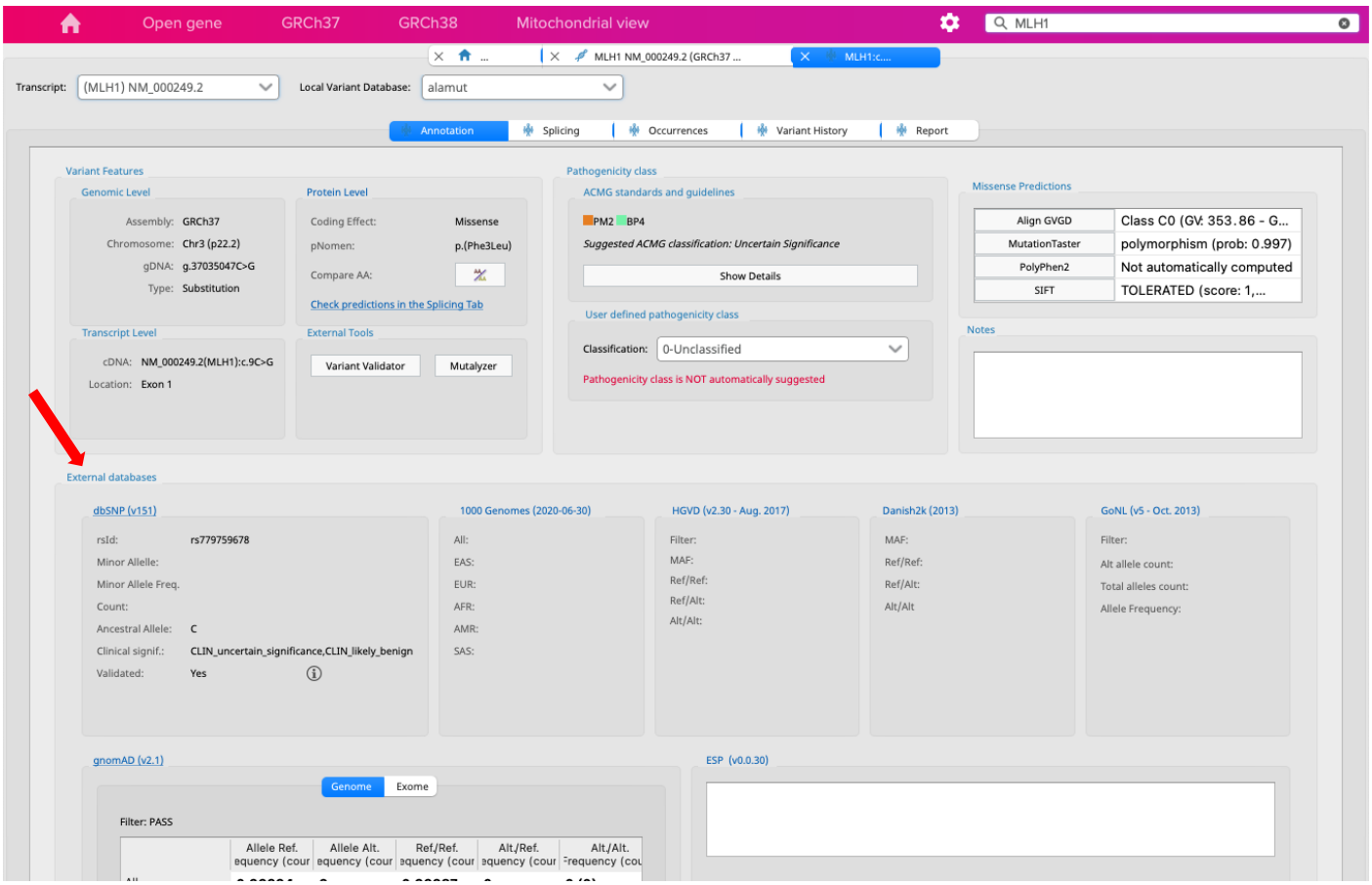




More information from external databases is available in Alamut® Visual Plus by right clicking and selecting the variant's gnomen:



The variant panel will pop-up with more information in the 'External databases' section:



References

- NCBI (Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine.)
- Genome Aggregation Database, gnomAD (The Broad Institute (URL: http://gnomad.broadinstitute.org).
- NHLBI GO Exome Sequencing Project (Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/)
- The Japan Human Genetic Variation Database, HGVD
- The Genome of the Netherlands Consortium, GoNL (Whole-genome sequence variation, population structure and demographic history of the Dutch population. Nature Genetics (2014) doi:10.1038/ng.3021.(URL: http://www.nlgenome.nl/).
- Variants and frequencies from the Whole-exome sequencing of 2,000 Danish individuals and the role of rare coding variants in type 2 diabetes (Whole-exome sequencing of 2,000 Danish individuals and the role of rare coding variants in type 2 diabetes. Am J Hum Genet. 2013 Dec 5;93(6):1072-86. doi: 10.1016/j.ajhg.2013.11.005. Epub 2013 Nov 2

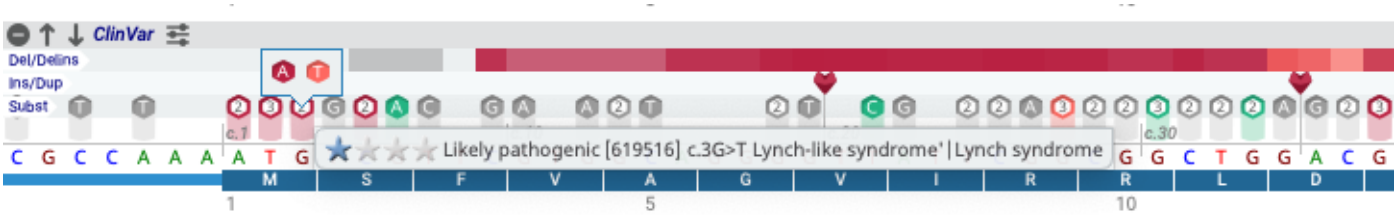
10.5.2. Clinvar

Alamut® Visual Plus displays ClinVar variants in a dedicated track
Variant background colors are based on the clinical significance provided by ClinVar submitters.

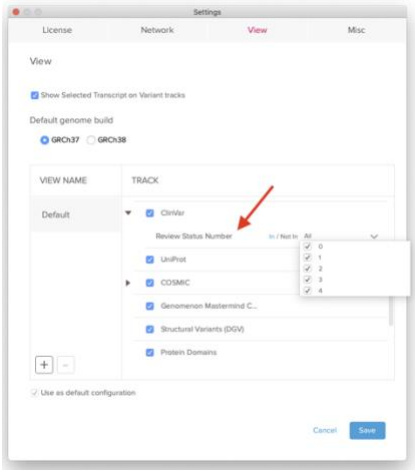
- **Red** for pathogenic variants
- **Orange** for likely pathogenic variants

- **Light Green** for likely benign variants
- **Green** for benign variants
- **Gray** for variants of an uncertain significance
- **White** for Unclassified variants

If multiple SNVs are reported at the same position, a tooltip pops-up to show significance for both variants.



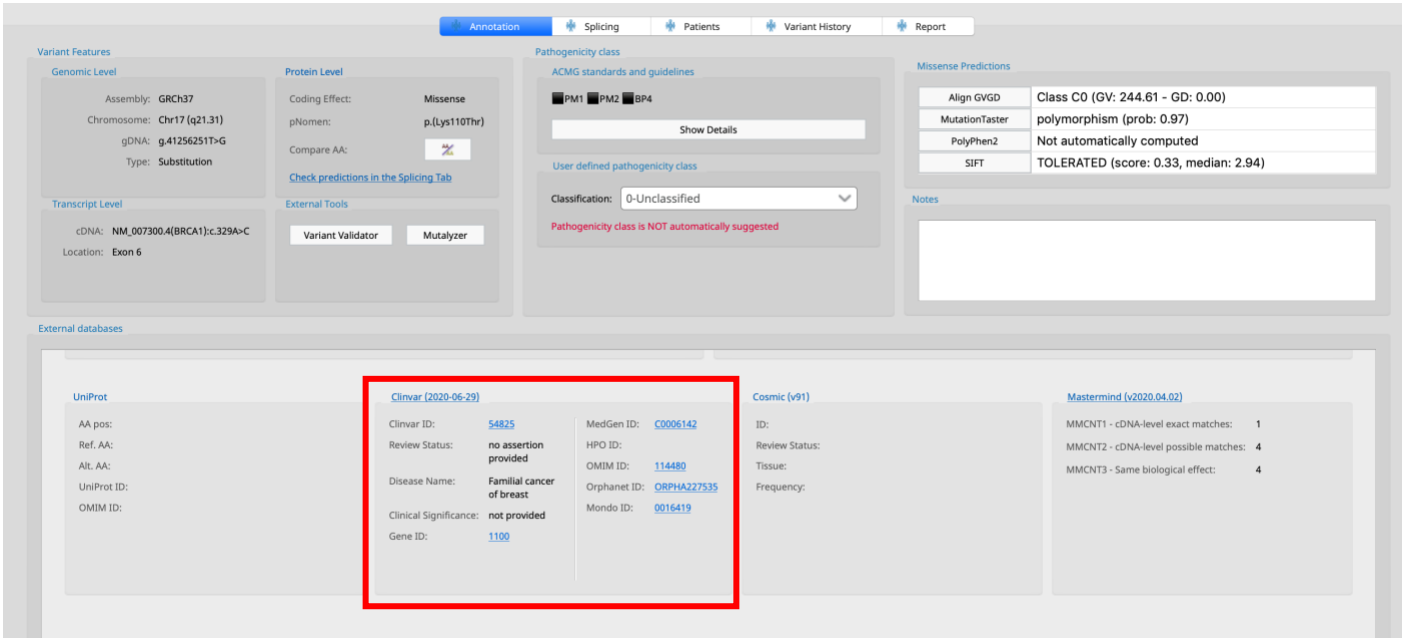
By clicking on 'Options' button from the ClinVar track, you can filter variants based on 'Review Status'.



The Review status numbers are defined as follows:

- **0** : No submitter provided an interpretation with assertion criteria (no assertion criteria provided), or no interpretation was provided (no assertion provided).
- **1 :** At least one submitter provided an interpretation with assertion criteria (criteria provided, single submitter) or multiple submitters provided assertion criteria but there are conflicting interpretations in which case the independent values are enumerated for clinical significance (criteria provided, conflicting interpretations).
- **2** : Two or more submitters provided the same interpretation (criteria provided, multiple submitters, no conflicts)
- **3** : Reviewed by expert panel.
- **4** : Practice guideline.

Right-clicking will show the link to the ClinVar website and to the Variant panel where information from ClinVar is displayed.

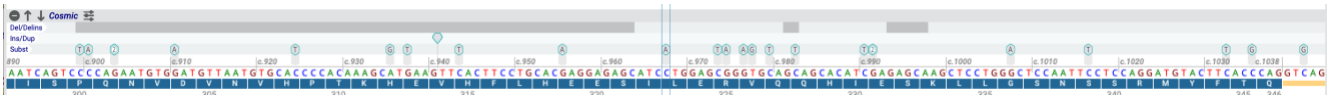


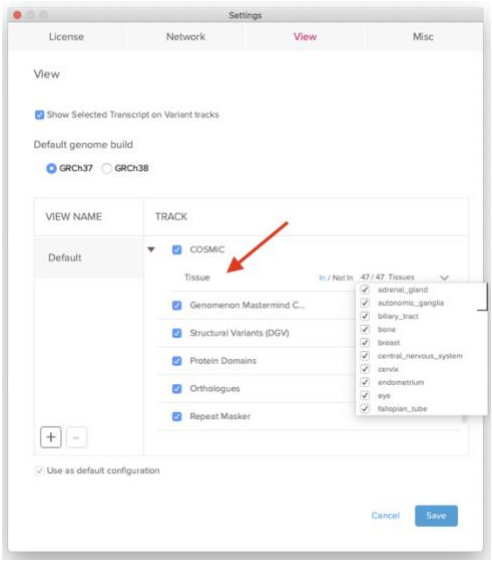
References

- Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, Maglott DR. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res. 2014 Jan 1;42(1):D980-5. doi: 10.1093/nar/gkt1113. PubMed PMID: 24234437
- Melissa Landrum, PhD, Jennifer Lee, PhD, George Riley, PhD, Wonhee Jang, PhD, Wendy Rubinstein, MD, PhD, Deanna Church, PhD, and Donna Maglott, PhD. ClinVar. NBK174587

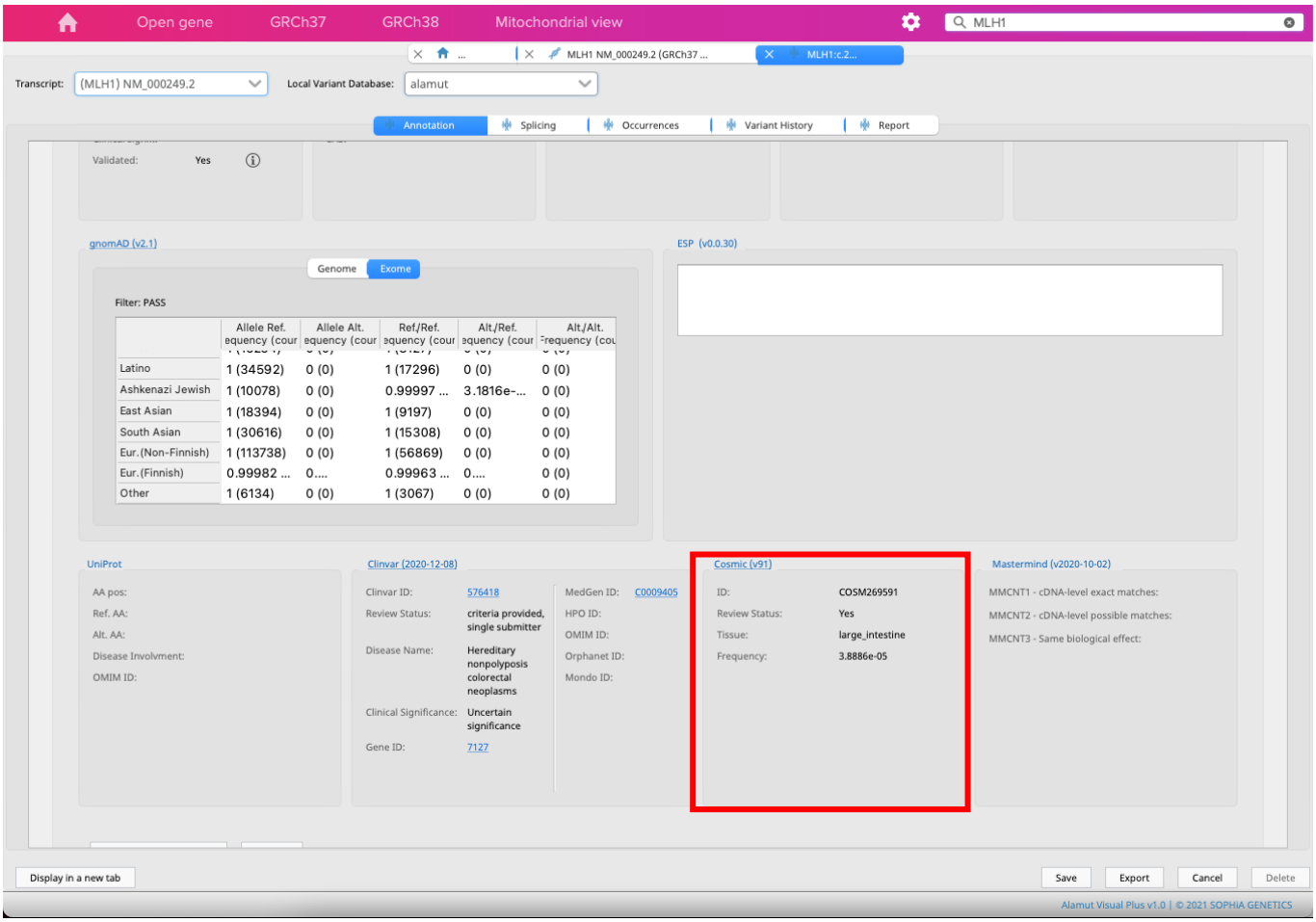
10.5.3. COSMIC

Alamut® Visual provides access to COSMIC variant in a separate track. Clicking on the 'Options' button via the COSMIC track allow to filter based on the 'Tissue' criteria.





Right clicking on a mutation opens the variant panel with information and directs you to the COSMIC website.



	Allele Ref.	Allele Alt.	Ref/Ref.	Alt/Ref.	Alt/Alt.
Latino	1 (34592)	0 (0)	1 (17296)	0 (0)	0 (0)
Ashkenazi Jewish	1 (10078)	0 (0)	0.99997 ...	3.1816e-...	0 (0)
East Asian	1 (18394)	0 (0)	1 (9197)	0 (0)	0 (0)
South Asian	1 (30616)	0 (0)	1 (15308)	0 (0)	0 (0)
Eur.(Non-Finnish)	1 (113738)	0 (0)	1 (56869)	0 (0)	0 (0)
Eur.(Finnish)	0.99982 ...	0....	0.99963 ...	0....	0 (0)
Other	1 (6134)	0 (0)	1 (3067)	0 (0)	0 (0)

The review status displayed in the variant panel are reported from COSMIC as following:

- The status "NA": “Not specified”, “Variant of unknown origin”, “Previously observed”
- The status “No”: "Reported in another sample as germline", "Confirmed germline variant"

- The status “Yes”: "Reported in another cancer sample as somatic", "Confirmed somatic variant"

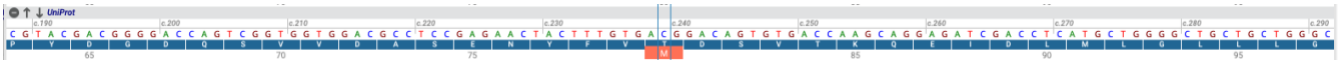
References

- Forbes et al. The Catalogue of Somatic Mutations in Cancer (COSMIC). Curr Protoc Hum Genet. 2008 Apr;Chapter 10:Unit 10.11.

- Forbes et al. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. Nucleic Acids Res. 2011 Jan;39(Database issue):D945-50

10.5.4. UniProt

The Protein Variant track displays variants set at protein level from UniProt. You can see the amino acid information and the disease involvement by hovering over a variant on the track. This information is also available in the variant panel.



References

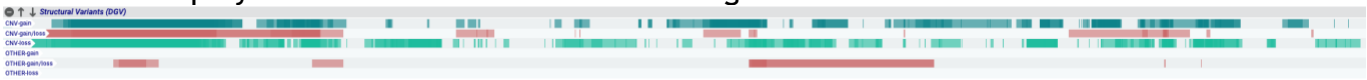
- UniProt: a worldwide hub of protein knowledge Nucleic Acids Res. 47:D506-515 (2019)

10.5.5. Protein Domain

The Protein Domains track will display the domain information from InterPro (via Ensembl).

10.5.6.DGV

The track displays the structural variations in the genome via the DGV database.



References

- MacDonald JR, Ziman R, Yuen RK, Feuk L, Scherer SW. The database of genomic variants: a curated collection of structural variation in the human genome. Nucleic Acids Res. 2013 Oct 29. PubMed PMID: 24174537

10.5.7. Repeat masker

The Repeat Masker track displays the repeat DNA regions identified by the Repeat Masker tool.

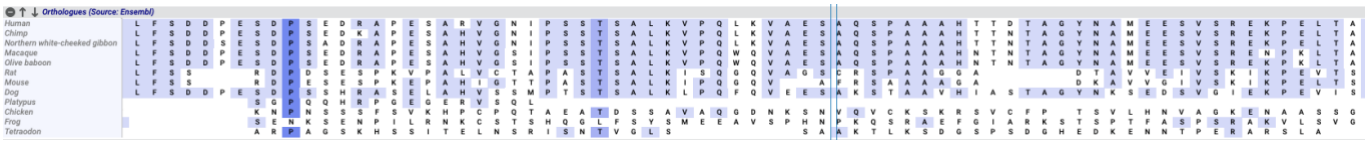
The regions are displayed in accordance to their type.

- SINEs
- LINEs
- DNA elements (DNA)
- Simple Repetitions (Simple Repeat)

- Low Complexity
- Satellite Elements (Satellite)
- Small RNAs (RNA)
- Not Classified (Unknown)
- Other

10.5.8. Orthologues alignments

The Orthologues track displays protein orthologue alignments from the Ensembl database, ICAR or IBC (In-house). These alignments show the conservation of amino acids across different species.



By default, orthologues aligned and displayed in Alamut® Visual are taken from the Ensembl Compara database. So far (March 2010), the only non-Ensembl-based alignments available were the manually-curated alignments of **ATM**NM_000051 and U82828, provided by Tavtigian et al. (2009) and **BRCA1** NM_007294, provided by IARC with Align GVGD.

References

- We would like to express our thanks to the Genetic Cancer Susceptibility Group at IARC for their kind help in defining our alignment protocol.
- Tavtigian, SV., Greenblatt, MS., Lesueur, F., Byrnes, GB. (2008). *In silico analysis of missense substitutions using sequence-alignment based methods*. *Hum Mutat*.11 : 1327-36
- Tavtigian, SV., Oefner, PJ., Babikyan, D. et al (2009). *Rare, evolutionarily unlikely missense substitutions in ATM confer increased risk of breast cancer*. *Am J Hum Genet*. 85 : 427-46.
- Deforche A., Blavier A. (2010). Systematic Building of Multiple Protein Alignments for Variant Interpretation *Human Genome Meeting* poster.

10.5.9. Genomenon Mastermind

Mastermind® by Genomenon® is a search engine with a comprehensive collection of genomic evidence and a user-friendly database.

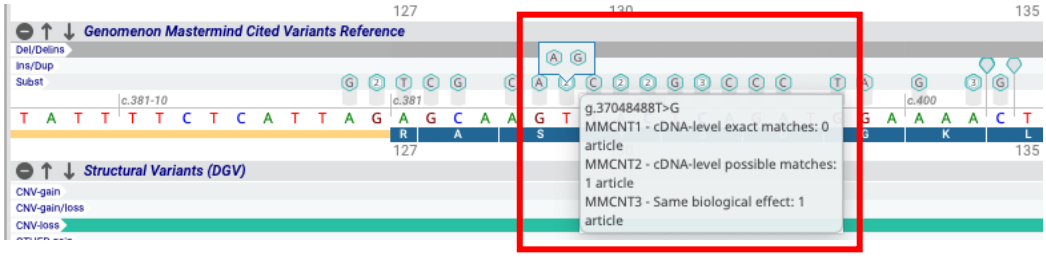
More information about Genomenon is available at https://www.genomenon.com/tutorials/
Mastermind visualization can be activated in the "Settings > View" tab for each view configuration.
Alamut® Visual Plus displays Mastermind variants in a dedicated track: Genomenon Mastermind Cited Variants Reference. Variants are reported from Cited Variants Reference (CVR), equivalent to a Variant Call Format (VCF) file. The CVR contains a count of articles associated with each

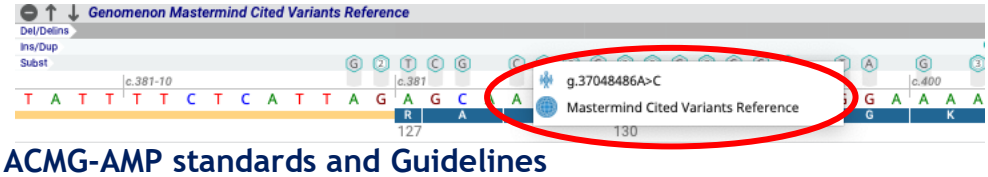
variant, along with a deep link into the related Mastermind UI. More information on CVR at https://www.genomenon.com/cvr/

If multiple SNVs are reported at the same position, a number indicating the substitutions occurring at this position. A pop-pup at the top of the position will display the different variations. Right-click on a variant to display links to the variant panel and to the Mastermind® website. A field in the variant panel displays the number of articles, including the three MMCNT values.

Mouse over on a CVR variant to pop-up a tooltip displaying the gnomen and the three MMCNT values:

- **MMCNT1: cDNA-level exact matches**
  The number of articles mentioning the variant at the nucleotide level in title/abstract or full-text.
- **MMCNT2: cDNA-level possible matches**
  The number of articles with nucleotide-level (from 1) and protein-level matching with not specified cDNA-level change (articles could refer to this nucleotide-level variant but there is insufficient data to determine conclusively).
- **MMCNT3: same biological effect matches**
  The number of articles citing any variant with the same biological effect as the considered variant. This includes MMCNT1 and MMCNT2 articles plus those with alternative cDNA-level variants that result in the same protein effect.



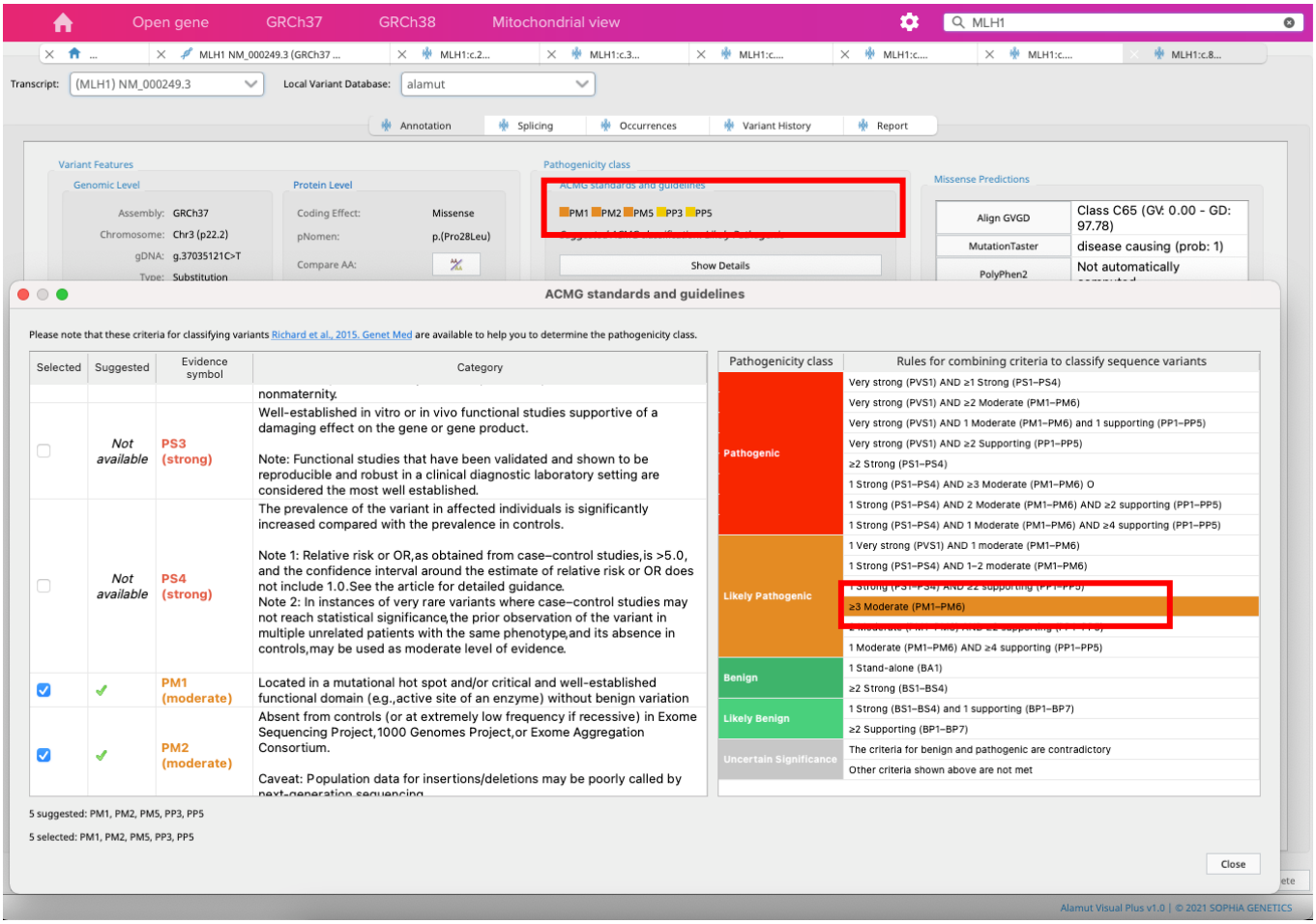


10.5.10. ACMG-AMP standards and Guidelines

ACMG-AMP Variant Interpretation Standards and Guidelines Support are available in Alamut® Visual Plus. The 2015 report from the American College of Medical Genetics and Genomics (ACMG) provides recommendations for the reporting and interpretation of sequence variants for Mendelian disorders in a clinical context (Richards et *al.*, 2015. *Genet Med* 17:405–424).
We provide automatic variant evidence based on external data available in Alamut database following guidance from Richards et *al.*, 2015.

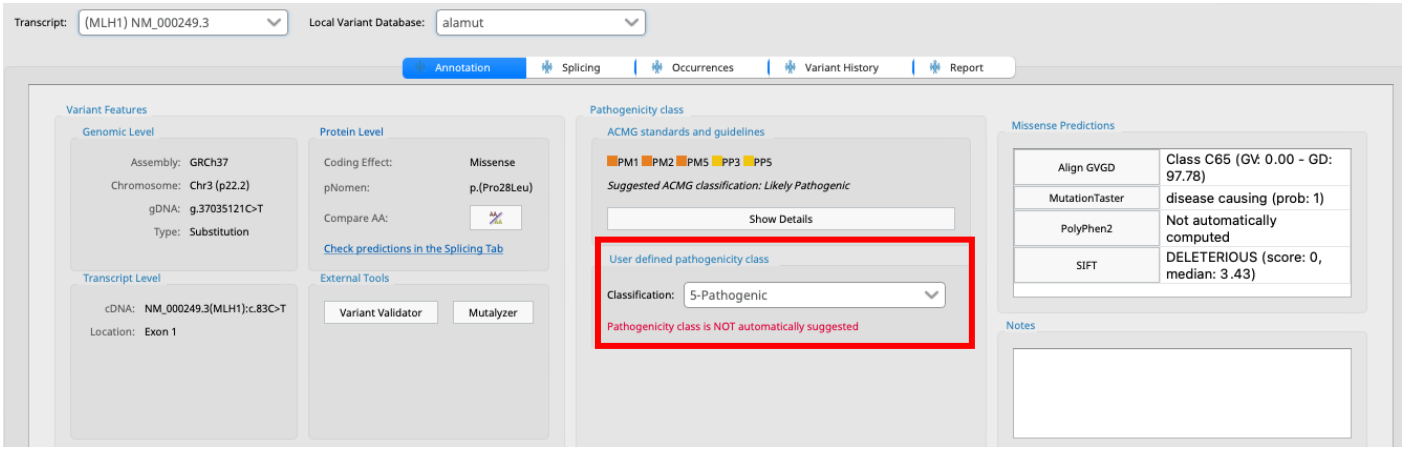
The ACMG guidelines support is available in the Variant panel > 'Annotation' tab > 'Pathogenicity Class' section.The suggested classification is displayed under 'ACMG standards and guidelines'.
By clicking in 'Show details', the user has access to definitions of categories as reported in Richards et al., 2015.

The user can modify variant evidence based on the scientific context. The selected evidence will then be displayed in the variant panel. The suggested ACMG pathogenicity class is assessed based on selected evidence by the user.



When data are not available in Alamut database to support an evidence, 'Not available' is displayed in the 'Suggested' field.
A blank/empty evidence field indicates that Alamut Visual Plus does not support these ACMG criteria, as the data required to determine these ACMG criteria is not available within Alamut Visual Plus (such as experimental results etc).

The final pathogenicity classification has to be defined by the user in the 'User defined pathogenicity class' section in the Variant panel.



10.6. In silico predictions

10.6.1. Splicing predictions

Alamut® Visual Plus includes a splicing module integrating a number of prediction algorithms and splicing prediction data:

- Splicing signals:
  - SpliceSiteFinder-like (donor, acceptor, branchpoint)
  - MaxEntScan (donor, acceptor)
  - GeneSplicer (donor, acceptor)
  - NNSPLICE (donor, acceptor)
  - Known constitutive signals (donor, acceptor)
  - Mercer et al. high-confidence branchpoints
- Exonic Splicing Enhancers (ESE) binding site detection:
  - ESEFinder
  - RESCUE-ESE
  - EX-SKIP

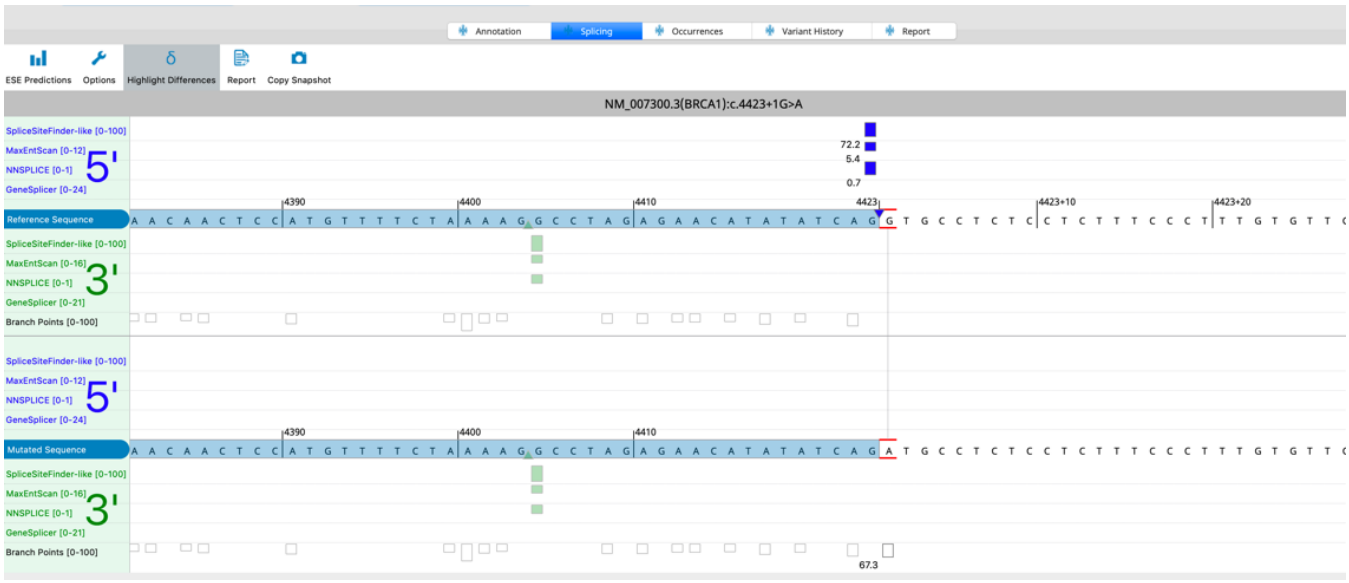
The splicing module includes:

- The variant annotation window with automatically computed splicing predictions at the nearest junction for MaxEntScan and SSF predictors.
- The splicing prediction algorithm NNSPLICE from fruitfly.org partially integrated: it can be interrogated from Alamut® Visual Plus through the Internet and its results are displayed seamlessly in the graphical interface.
- The splicing report that provides scores for each predictor in a tabular format.

To open the splicing window, click on the 'Splicing' tab in the variant panel:

The window displays the reference (wild-type) and mutated sequences and predictions are reported above and under each one. Exons are drawn as blue boxes. Hits from SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer are displayed as blue vertical bars for 5' (donor) sites, and as green vertical bars for 3' (acceptor) sites. The height of each bar is proportional to the maximum possible score computed by the corresponding algorithm. Known constitutive signals are displayed as small blue (5') or green (3') triangles, close to the sequence letters.
Mercer et al. high-confidence branchpoints are displayed as red triangles in the Branch Points sub-track of the Reference Sequence.

When moving the mouse over each vertical bar or triangle, a tooltip appears with the corresponding score. You can display score numbers for each hit bar by just clicking the bar itself.



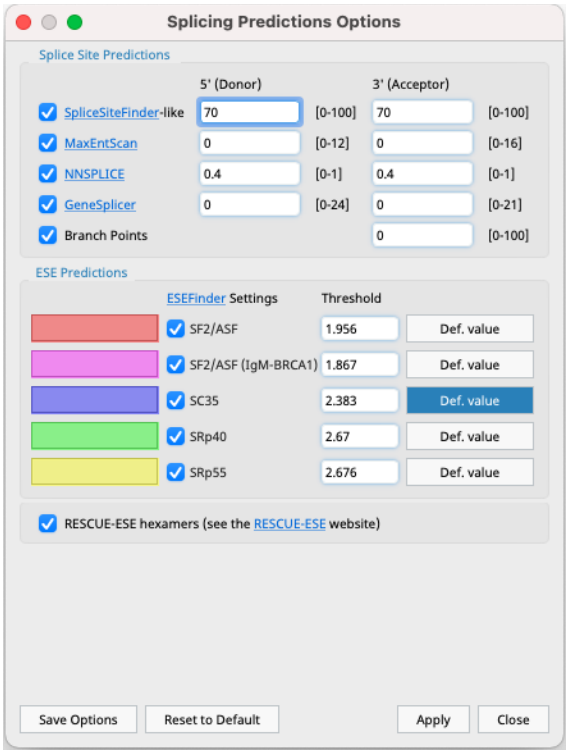
The region for which predictions are computed is centred on the position of the variant. You can navigate before and after this region by clicking and dragging the mouse.
Use the “Options” menu to select which predictions to display and to modify thresholds.

To reveal differences between wild-type and mutated scores, click on the '**Highlight Differences**' button. Unchanged hits get dimmed, while scores are displayed beside those that differ.
To reveal differences between wild-type and mutated scores, click on the '**Highlight Differences**' button. Unchanged scores get dimmed, while score numbers are displayed beside those that differ.
To display ESE predictions, click the "ESE Predictions" button. ESE hits from ESEfinder are displayed above each sequence, and RESCUE-ESE hexamers are drawn under them:

To launch the EX-SKIP tool, click the "EX-SKIP" button. The "EX-SKIP" button is displayed after clicking the "ESE predictions" button). The pre-filled web form of the EX-SKIP tool is displayed in a new window. Input sequences are created as follows by Alamut Visual Plus: only exonic sequences are taken into account with up to 30 exonic nucleotides before or after the variant position within the exon.

To generate a tabular report of splicing signals predictions, click the 'Report' button. By right clicking on the report you can save it in your computer. The flanking region threshold can be set by the user. The default is fixed at 0.

The user can select displayed splicing methods from the “Options” menu. Thresholds for splice site predictors can be saved as user-defined parameters.



Background on prediction methods:

SpliceSiteFinder-like

This method is based on position weight matrices computed from a set of human constitutive exon/intron junctions for donor (both GT and GC) and acceptor sites.
Alamut® Visual Plus uses the matrix described by Zhang et al. (1998) for branch points and the algorithms described in Shapiro et al. (1987).

MaxEntScan

MaxEntScan is a method based on the Maximum Entropy principle, developed by the Burge Lab at MIT and described in Yeo et al., 2004. The MaxEntScan splice site datasets and algorithms are fully integrated inside Alamut® Visual Plus, with permission from Christopher Burge.
Alamut® Visual Plus only reports scores from the Maximum Entropy Model.

NNSPLICE

NNPLICE (available at the Berkeley Drosophila Genome Project web site) is a prediction method based on neural networks (Reese et al. 1997). Although not fully integrated inside Alamut® Visual Plus, it is transparently queried from the software.
Alamut® Visual Plus reports scores from NNSPLICE 0.9.

GeneSplicer

GeneSplicer is an Open Source software available from the University of Maryland CBCB.
GeneSplicer combines several splice site detection techniques, among which Markov models (Pertea et al. 2001).

Known constitutive signals

Alamut® Visual Plus reports in the splicing module each occurrence of the 9-mers (3 exonic + 6 intronic nucleotides) found in the donor subset of human constitutive exon/intron junctions, and each occurrence of the 6-mers (4 intronic + 2 exonic) found in the acceptor subset. Acceptor 6-mers are reported only where at least 6 of the 8 upstream nucleotides are pyrimidines.

Mercer et al. high-confidence branchpoints

Using exoribonuclease digestion and targeted RNA-sequencing to enrich for sequences that traverse the lariat junction and, by split and inverted alignment, Mercer et al. (2015) identified 59,359 high-confidence human branchpoints in >10,000 genes, thus providing a first map of splicing branchpoints in the human genome.

ESEFinder

The ESEFinder method computes putative binding sites for Exonic Splicing Enhancers (Cartegni et al., 2003). We have embedded the ESEFinder matrices (licensed from Cold Spring Harbor Laboratory) inside Alamut® Visual Plus so as to perform the same computation as that provided by the CSHL ESEFinder web site.

RESCUE-ESE

In the RESCUE-ESE approach, specific hexanucleotide sequences are identified as candidate ESEs (Fairbrother et al., 2002). The set of human hexamers available from the RESCUE-ESE web site is

embedded inside Alamut® Visual Plus.

EX-SKIP

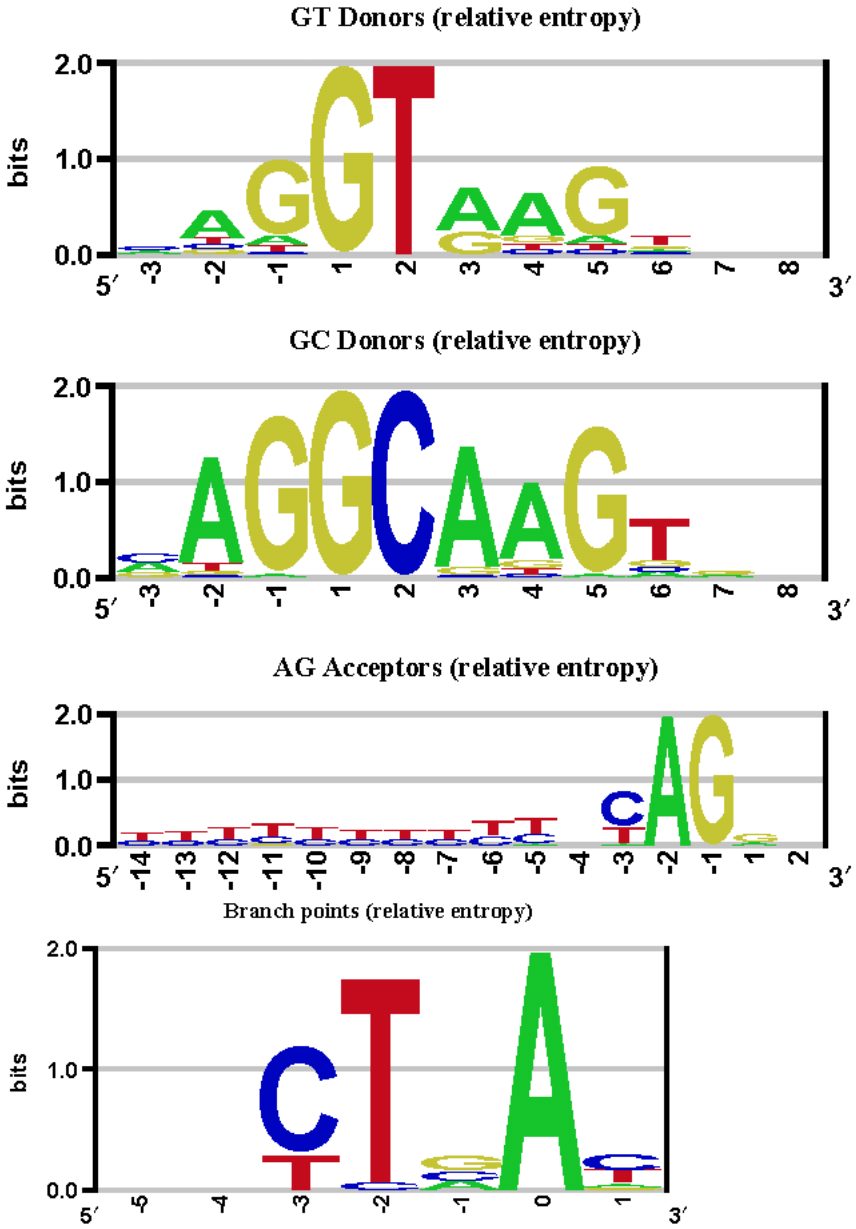
EX-SKIP compares the ESE/ESS profile of a wild-type and a mutated allele to quickly determine which exonic variant has the highest chance to skip this exon. It calculates the total number of ESSs, ESEs and their ratio. Specifically, it computes the number of RESCUE-ESEs (Fairbrother et al., 2004; Fairbrother et al., 2002), FAS-ESSs (Wang et al., 2004), PESEs/PESSs (Zhang et al., 2004), neighborhood inference (Stadler et al., 2006) and EIE/IIEs (Zhang et al., 2008) for each segment.
The EX-SKIP tool is available, through the Alamut® Visual pre-filled form functionality, from the menu bar of the "Splicing Effects" window after displaying ESE predictions.

Set of human constitutive exon/intron junctions

We have gathered a set of human constitutive exon/intron junction sequences as follows. 10,728 human mRNA sequences from the RefSeq database (as of Dec. 2007), with status 'reviewed', were mapped onto the human reference genome (NCBI 36). Based on this mapping, genomic exon/intron boundary sequences were extracted into separate subsets for donor and acceptor sites.
With these sequences, we have built three position weight matrices: two matrices for donor sites (GT and GC sites), and one matrix for acceptor sites (AG sites).

Sequence logos

These sequence logos depict the position weight matrices used by the SpliceSiteFinder-like algorithm in Alamut® Visual Plus.



References

Cartegni et al. *ESEfinder: A web resource to identify exonic splicing enhancers*.
Nucleic Acids Res (2003) vol. 31 (13) pp. 3568-71
Fairbrother et al. *Predictive identification of exonic splicing enhancers in human genes*.
Science (2002) vol. 297 (5583) pp. 1007-13
Hellen *Splice Site Tools: A Comparative Analysis Report*.
NGRL Manchester Report 2009.
Houdayer et al. *Guidelines for splicing analysis in molecular diagnosis derived from a set of 327 combined in silico/in vitro studies on BRCA1 and BRCA2 variants*.
Hum Mutat. 2012 Aug;33(8):1228-38.
Mercer at al. *Genome-wide discovery of human splicing branchpoints.*
Genome Res (2015) 25(2): 290-303.

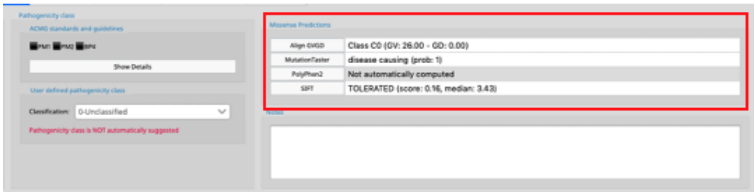
Pertea et al. *GeneSplicer: a new computational method for splice site prediction*.
Nucleic Acids Res (2001) vol. 29 (5) pp. 1185-90
Raponi, M., Kralovicova, J., Copson, E., et al. *Prediction of single-nucleotide substitutions that result in exon skipping: identification of a splicing silencer in BRCA1 exon 6.*.
Hum Mutat. (2011), 32, 436-444.
Reese et al. *Improved Splice Site Detection in Genie*.
J Comp Biol (1997) vol. 4 (3), pp. 311-23
Shapiro, M. B. and P. Senapathy (1987). *RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression.*
Nucleic Acids Res 15(17): 7155-7174.
Yeo et al. *Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals*.
J Comput Biol (2004) vol. 11 (2-3) pp. 377-94
Zhang et al. *Statistical features of human exons and their flanking regions*.
Hum Mol Genet (1998) vol. 7 (5) pp. 919-32

10.6.2. Missense predictions

Alamut® Visual Plus provides automatically computed missense predictions done by Align GVGD and SIFT, based on orthologues alignments (see section 10.5.8. Orthologues alignments).
With the genome build GRCh38 available in Alamut® Visual Plus, some orthologues alignments coming from ENSEMBL Compara have been updated and may lead to different predictions that those described for the same genes/transcripts for GRCh37.
Currently, automatically computed predictions are provided for Align GVGD, SIFT and MutationTaster.

Automatically computed predictions are available in the variant panel. Polyphen2 scores are not automatically computed.

Clicking on the predictor name will bring you to the missense predictor website.



This section describes how automatically computed missense predictions are provided in Alamut® Visual Plus:

Align GVGD
Align GVGD predictions are computed using the orthologue alignment provided by Alamut® Visual Plus for the gene under study (See section 10.5.8. Orthologues alignments).
Align GVGD scores interpretation are available here.

Current version for precomputed predictions: Align GVGD Tavtigian et al. (2006)

SIFT
SIFT predictions are computed using the orthologue alignment provided by Alamut® Visual Plus for the gene under study. See SIFT Aligned Sequences.
SIFT scores interpretation are available here.
Current version for precomputed predictions: SIFT 6.2.0

MutationTaster
MutationTaster predictions are computed using the mapping between NCBI RefSeq and Ensembl Transcript Ids available in Alamut® Visual plus.
MutationTaster scores interpretation are available here.
Current version for precomputed predictions: MutationTaster2 from MutationTaster website

References
NGRL Manchester *Multiple sequence alignment use in missense prediction tools*

Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A. *Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral*. J Med Genet. 2005 Jul 13

Ng PC, Henikoff S. *Predicting Deleterious Amino Acid Substitutions*. Genome Res. 2001 May;11(5):863-74. PubMed

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. *A method and server for predicting damaging missense variants*. Nat Methods 7(4):248-249 (2010)

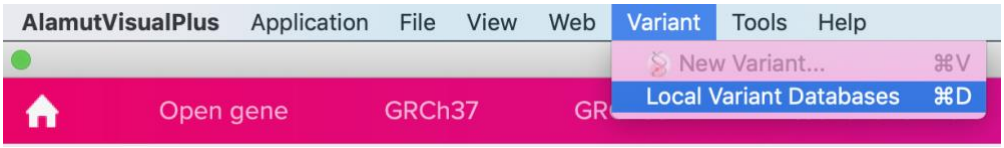
Schwarz JM, R&#xfffd;delsperger C, Schuelke M, Seelow D. *MutationTaster evaluates disease-causing potential of sequence alterations*. Nat Methods. 2010 Aug;7(8):575-6.

Special thanks to the Genetic Cancer Susceptibility Group at IARC for their kind help in implementing the Align GVGD algorithm.

10.7. Managing variants

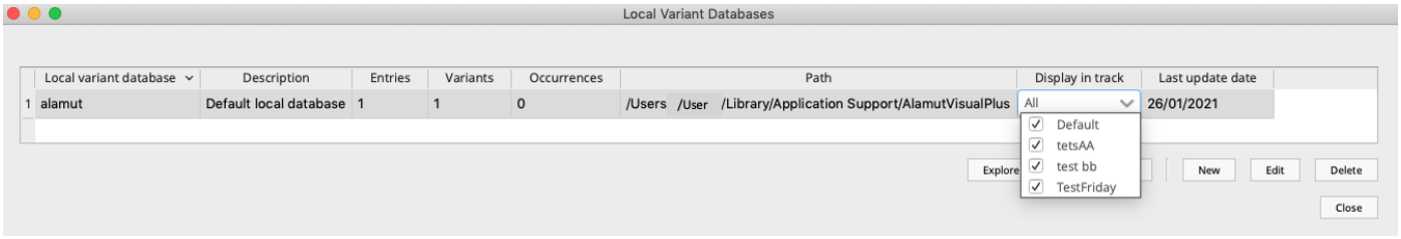
10.7.1. Entering variants

Local Variant database
Alamut® Visual Plus allows you to annotate and store variants handled through the software.
Click on menu > Variant > Local Variant Databases.



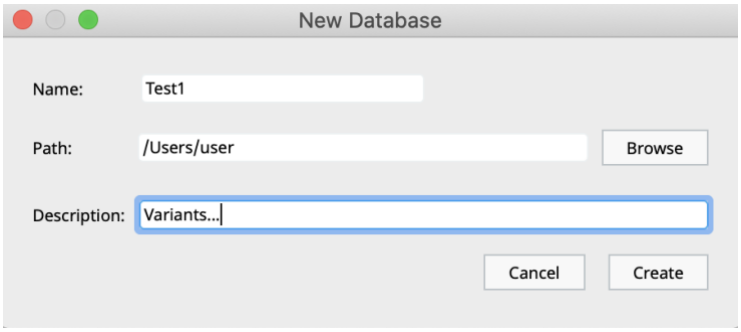
By default, a local database called “Alamut” is available, you can use it to store your internal variants. By selecting the ‘Display in track’ checkbox, you will be able to see your Local Variant Database and its variants in a separate new track in the interface. From the ‘Display in track’

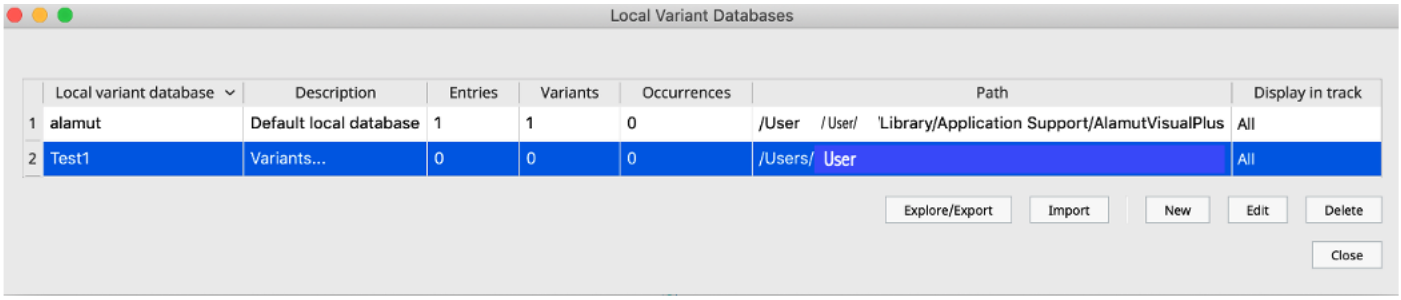
check box, you can select the 'View configuration' where you can see the new track. You can see several Local Variant Databases at one time.



By clicking in 'New' in the Local Variant Databases, a new tab will appear 'New Database'. You can define a new database by selecting a path, adding a name and a description. You can easily switch from one database to another.

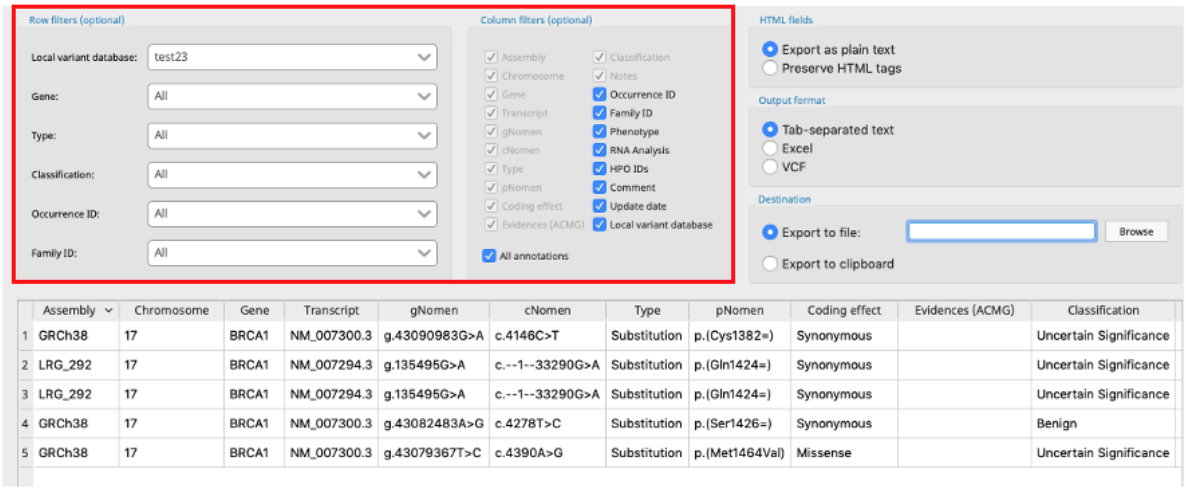
The basic variant database functionality implementation allows to store databases on each user's computer, or on a shared folder. When variants are stored in a shared file system, caution must be taken so that two people don't edit concurrently variants.





Alamut® Visual Plus enables also to list and navigate easily through all internal variants for the current gene by selecting a database and clicking in 'Explore/Export' button in the 'Local Variant Databases' tab.

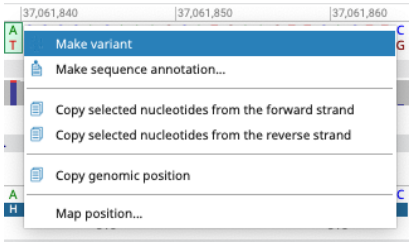
Variants will be displayed in a new tab 'Variant exporter'. You can display different options as showed in the 'Row filters' and 'Column filters' fields. You can scroll down/up and right/left to explore your internal variants.



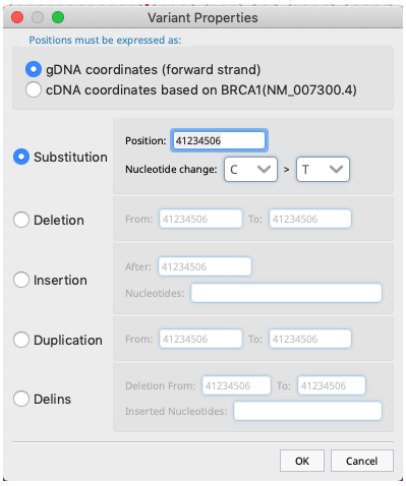
	Assembly	Chromosome	Gene	Transcript	gNomen	cNomen	Type	pNomen	Coding effect	Evidences (ACMG)	Classification
1	GRCh38	17	BRCA1	NM_007300.3	g.43090983G>A	c.4146C>T	Substitution	p.(Cys1382=)	Synonymous		Uncertain Significance
2	LRG_292	17	BRCA1	NM_007294.3	g.135495G>A	c.-1-33290G>A	Substitution	p.(Gln1424=)	Synonymous		Uncertain Significance
3	LRG_292	17	BRCA1	NM_007294.3	g.135495G>A	c.-1-33290G>A	Substitution	p.(Gln1424=)	Synonymous		Uncertain Significance
4	GRCh38	17	BRCA1	NM_007300.3	g.43082483A>G	c.4278T>C	Substitution	p.(Ser1426=)	Synonymous		Benign
5	GRCh38	17	BRCA1	NM_007300.3	g.43079367T>C	c.4390A>G	Substitution	p.(Met1464Val)	Missense		Uncertain Significance

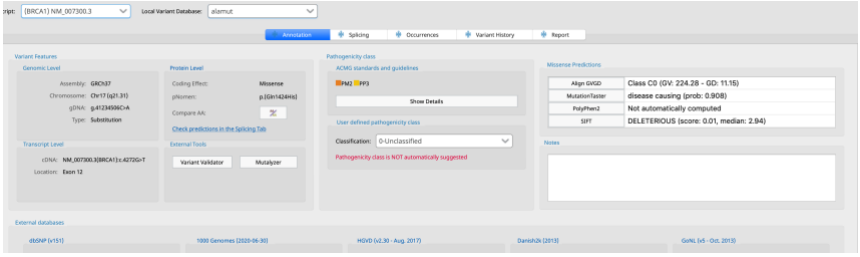
Creating a variant

You can manually create variants either from the 'Variants' menu, or from a nucleotide selection in the genome or transcript track and its associated context menu (by right-clicking inside the selection).



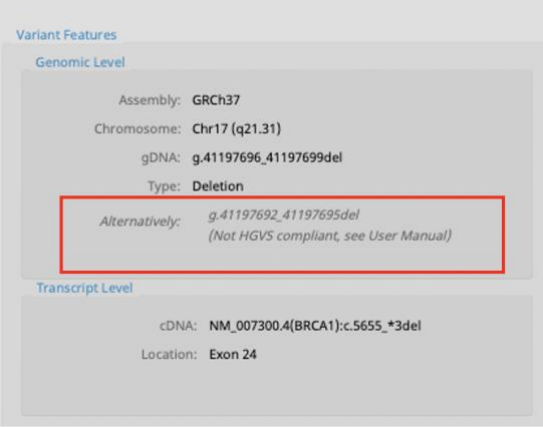
Once you have specified variant basic properties (position and type of change), the ‘Variant Panel’ (annotation window) opens in a new tab. In the ’Variant properties’ you can choose to apply your variant at the genome or transcript level.





Notes : **Application of HGVS recommendations**

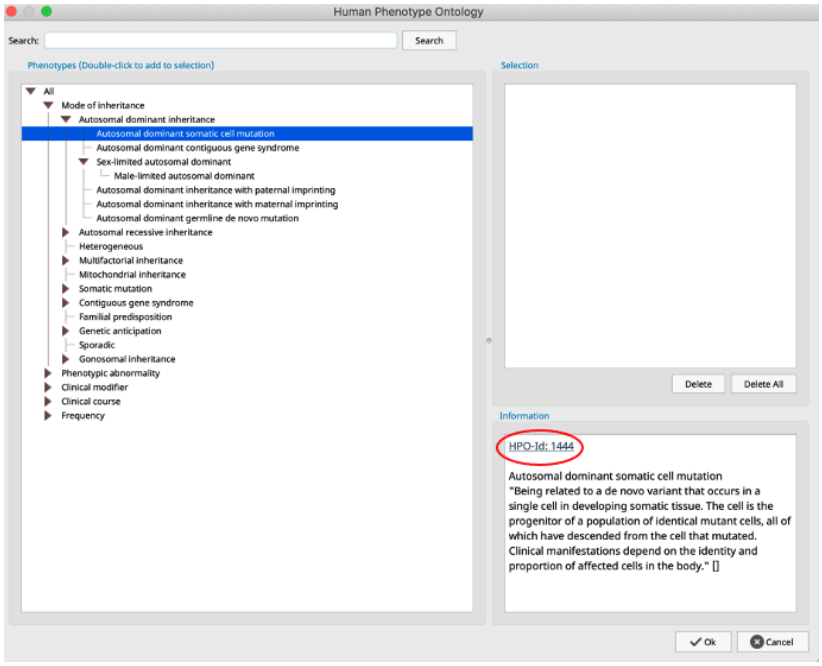
- Alamut® Visual Plus allows you to check in one click your variant with Mutalyzer Name Checker or VariantValidator from the annotation window
- The HGVS recommendations for the description of sequence variants implies that for all descriptions the most 3' position possible is arbitrarily assigned to have been changed. Application of this recommendation can make variant entering in Alamut® Visual Plus sometimes disconcerting, since entering a variant may result in a variant located at a different position. The point is that both mutated sequences – entered and resulted – are identical.
- The 3' rule is applied at the genome level. So it is indicated the most 3' position possible according to the forward sequence, and at the cDNA level so it is indicated the most 3' position possible according to the transcript. Thus, in reverse strand, the cDNA-level variant positions do not always map to the same genome-level positions (this is no compliant with HGVS (*For Alamut Visual users: this is handled differently as compared to Visual. In the Variant Panel > Variant Features > Genomic Level > alternatively; it is indicated the HGVS nomenclature as referred to Visual*)



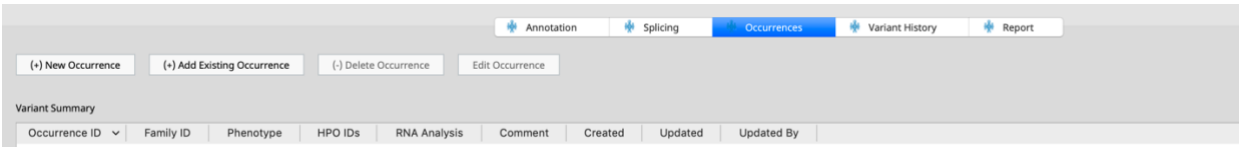
In the 'Variant Panel', you can find 5 sub tabs:

1. Annotation. Provide the external annotation of a variant (see section 10.5, for each catalog description)
2. Splicing. Provides splicing predictions scores (see section "10.6.1. Splicing predictions" for more details)
3. Occurrences. You can record different occurrences of the same variant. To create an occurrence, in the 'Occurrences' sub-tab, click on the 'New Occurrence' button. In the 'Edit Occurrence' tab panel, you can then enter specific information about the Occurrence. Fields RNA Analysis, Phenotype and Comment are enabled.

By clicking in the 'see HPO' button. You can select a phenotype for your occurrence as referred to the HPO. A link to the HPO website is also provide. By double clicking on a phenotype, the selection field will be filled in. The HPO-IDs will be reported in the final 'Occurrences' sub-tab.



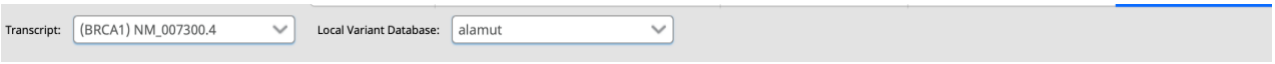
The entered information related to the occurrence are shown in a table in the 'Occurrences' sub-tab.
You can then manage your occurrences by deleting, editing or adding existing occurrences:



4. Variants history. Provides a history related to a same variant when updates are provided. The variant history includes the 'Date', the 'User', the final pathogenicity 'Classification' and the Notes as written in the 'Annotation sub-tab.

5. Report. Report the investigation related to one variant (See section 10.7.4. Reporting Variants)

Finally, you can save your created variant.
You can choose to modify your 'Transcript' and 'Local Variant Database' using the menu on the Top of the 'Variant Panel':



Note: In a same Local Variant Database, you cannot save same variant with different transcripts.

Variants are graphically displayed in tracks based on the Local Variant Database where the variant is saved (See.10.7.1. Entering variants). The color of variant graphic items depends on pathogenicity annotations. By default:

- Unclassified (gray),
- Benign (dark green),
- Likely benign (light green),
- Uncertain significance (blue),
- Likely pathogenic (Orange)
- Pathogenic (red).

10.7.2. Importing variants

Supported file format
Alamut® Visual Plus can import variant from external sources to populate the Local Variant Database. The imported file will be affected to the selected LVD.

When variants are successfully imported, they are saved as standard Alamut® Visual Plus internal variants and can then be handled like other internal variants.
Alamut® Visual Plus can import variant annotations from TSV (Tab-separated values) formatted files as Variant Calling Format files (VCF).

Note that for Alamut® Visual users, the same workflow can be used to import .mut files and .txt variants files that may have been exported from Alamut® Visual.

TSV format

Alamut® Visual Plus accepts a tabular import file containing variant descriptions and annotations. Import files must follow a precise format that is most easily created using a spreadsheet application like Excel. Here is an example. The column order must be strictly observed.

The header line (with column labels) is mandatory. Columns must be named as in the example in below:

Assembly	Chromosome	Gene	Transcript	gNomen	cNomen	Type	pNomen	Coding effect	Evidences (ACMG)	Classification	Notes	Occurrence ID	Family ID	Phenotype	RNA Analysis	HPO IDs	Comment
GRCh37	2	MSH2	NM_000251.2	g.47630335C>A	c.5C>A	Substitution	p.(Ala2Glu)	Missense	PM1,PM2,PP3	Likely Benign	This is a note relating to this variant	1	123	Adam+			
GRCh37	2	MSH2	NM_000251.2	g.47630347_47630350del	c.17_20del	Deletion	p.(Lys6Argfs*57)	Frameshift		Likely Pathogenic		2	456				
GRCh37	2	MSH2	NM_000251.2	g.47657084A>C	c.1276+4A>C	Substitution	p.?			Undefined Significance							

The following sets of columns must be populated in order to define the variants:

- Assembly
- Chromosome
- gNomen

or

- Gene
- Transcript
- cNomen

Assembly — GRCh37 or GRCh38.
Chromosome — chr**N** or **N,** where N is the number of the chromosome (or X or Y, or MT)
Gene — The official symbol of the gene carrying the variant.
Transcript — The RefSeqDna of the transcript used to describe the variant.
gNomen — The gDNA-level variant description, using the HGVS nomenclature.
cNomen — The cDNA-level variant description, using the HGVS nomenclature.
Type — The variant type: Substitution, Deletion, Insertion or Duplication
pNomen — Consequence of the variant at the protein level
Coding Effect — Missense, Start loss, Nonsense, Stop loss, Frameshift, In-frame or Synonymous
Evidences (ACMG) — ACMG evidence, separated by coma.
Classification — The variant classification:

- Benign
- Likely Benign
- Uncertain Significance
- Likely Pathogenic
- Pathogenic
- Undefined Significance

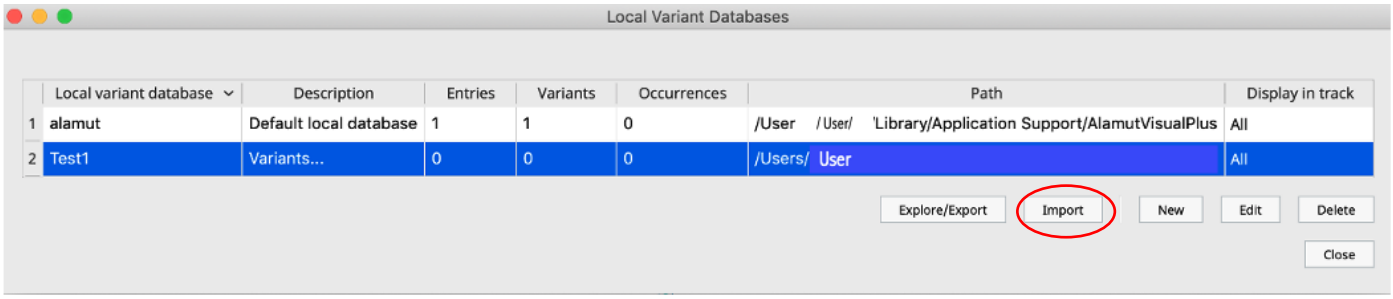
If no value is supplied here, " Undefined Significance" is assumed
Notes — Comments associated to the variant. Free content field.
Occurrence ID — Free content field.
Family ID — Free content field.
Phenotype — Free content field.
RNA Analysis — Free content field.
HPO IDs — Human phenotype ontology IDs
Comment — Comment associated to the occurrence. Free content field.

VCF format

Standard VCF files can be imported. Fields are #CHROM POS ID REF ALT QUAL FILTER INFO FORMAT
Only CHROM, POS, REF and ALT are used to set up the variant.

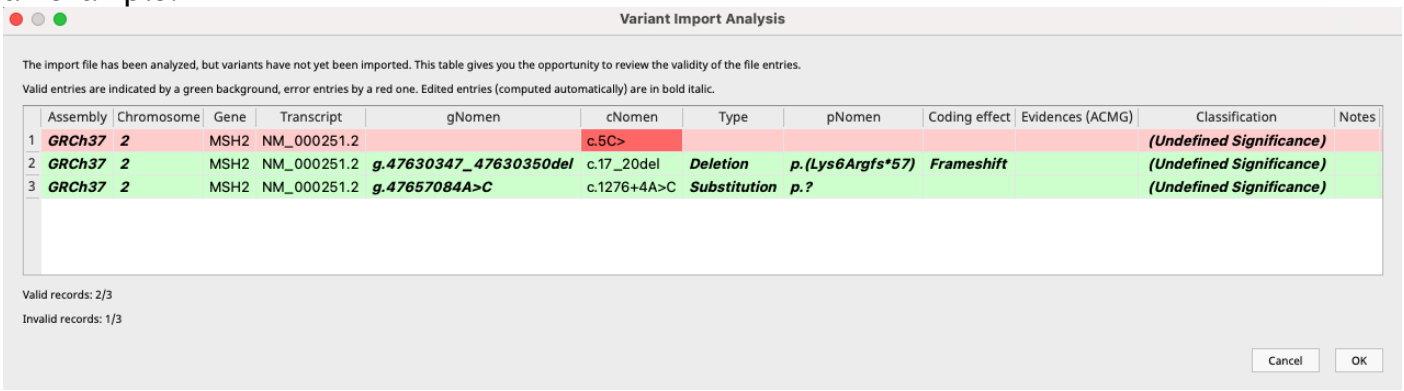
The import process

To import variants from an import file as described above, in Alamut® Visual Plus: Menu > 'Variants' > 'Local Variants Databases'. Select a line (Local Variant Database) where the variants will be stored and specify the import file you have by clicking on 'Import'.



Alamut® Visual Plus will analyse the imported file and reports valid and invalid entries based on the format.

Only validated variants (shown in green) will be imported to the Local Variant Database. Here is an example:



Notes:

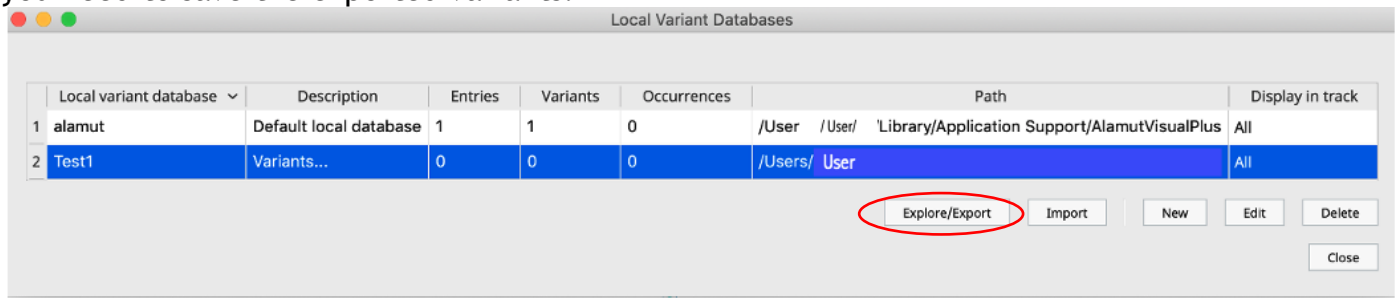
- The import process can be managed without opening a specific gene or transcript from the homepage of Alamut® Visual Plus
- The import process can also be managed when a gene and a transcript are already selected and opened.
- For VCF and TSV (with gNomen) import files, variants outside of the gene locus will also be processed.
- To visualize variants in tracks, you need to open the appropriate gene and transcript. The 'Display track?' in the Local Variant Databases tab should be selected (see section 10.7.1. Entering variants).

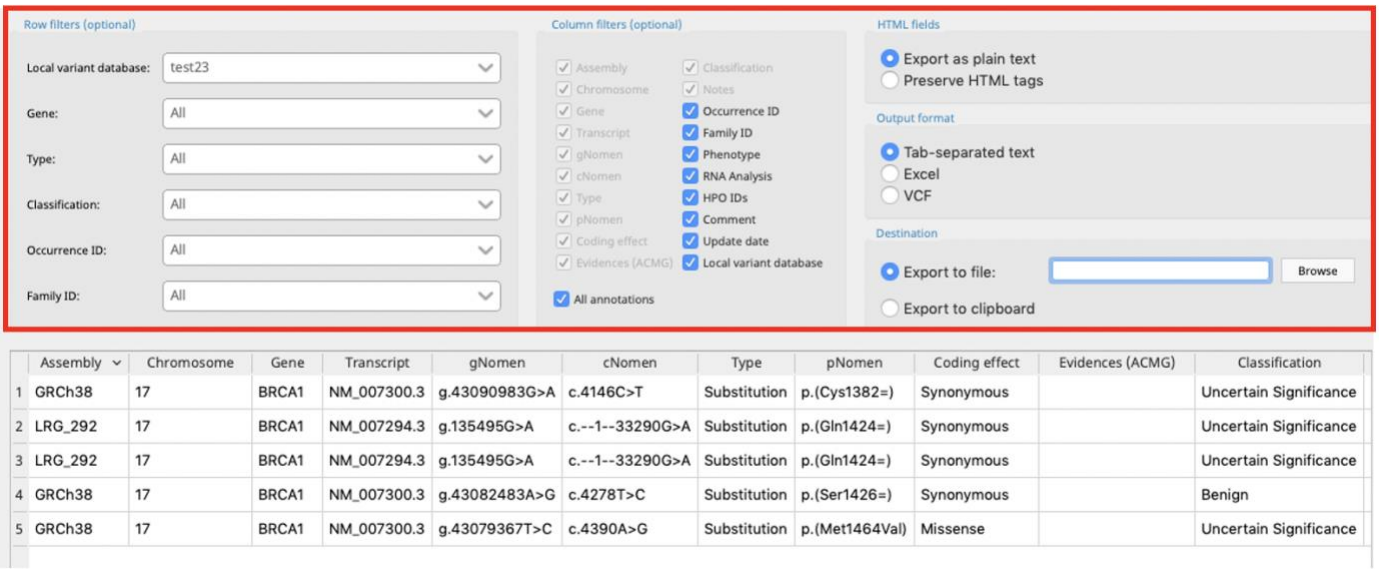
10.7.3. Exporting variants

Export internal Variants

To export variants already entered in Alamut® Visual Plus to Excel, to tab-delimited text files or to VCF, go to menu 'Variants' > 'Local Variants Databases' and Select the source Local Variant Database.

The 'Variant Exporter' pops-up. Select options, fields you need to export and the folder where you need to save the exported variants.





All grayed fields in the 'Column filters' are exported by default.

Export Variants with external annotation

For each variant entered in the software, Alamut® Visual Plus generates a set of annotations. Annotations are gathered for each variant and can be exported by ticking the "All annotations" checkbox.

10.7.4. Reporting Variants

You can create a final report including all information related to one specific variant. This functionality is available via the 'Variant Panel' > 'Report' sub-tab.

In the menu on the right, you can select features to display in the final report.
By right clicking in the report, you can save the report in a PDF or HTML format.

10.8. Data visualization

10.8.1. Viewing BAM/CRAM alignment files

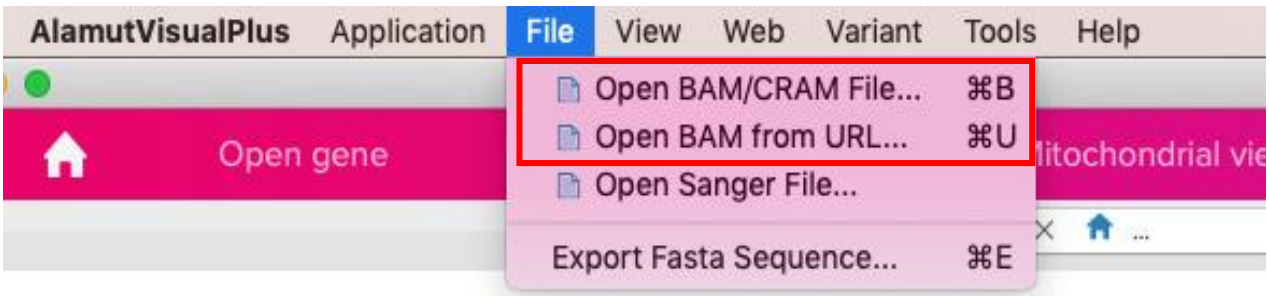
Alamut® Visual Plus allows to visualize alignment from BAM and CRAM files.

Loading BAM and CRAM files

Two options are available to load alignment files once your gene of interest is opened:

- Select menu "File > Open BAM/CRAM File" to load one or more BAM or CRAM files from your computer or local network.
- Select menu " File > Open BAM from URL" to load a BAM file from a web server. HTTP and HTTPS protocols are supported.

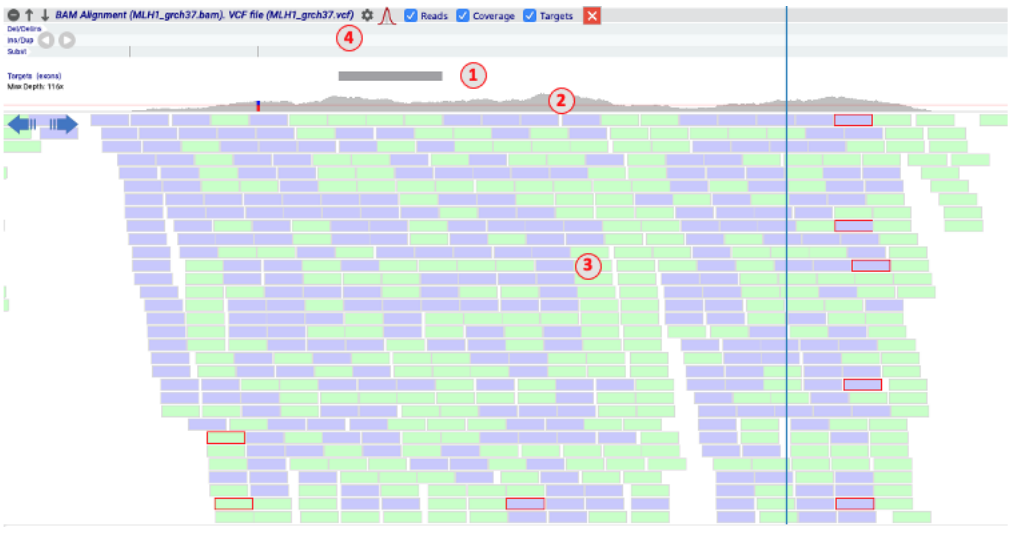
Using CRAM files usually requires defining the location of reference sequences. (See Miscellaneous)



Note: One or multiple alignments can be loaded for one gene.

BAM Viewer components

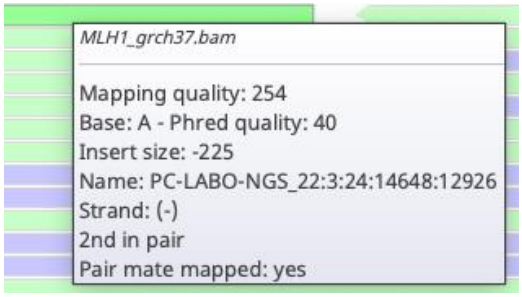
The BAM track includes different components



1. Sequencing targets
2. Depth of coverage
3. Reads
4. Variants

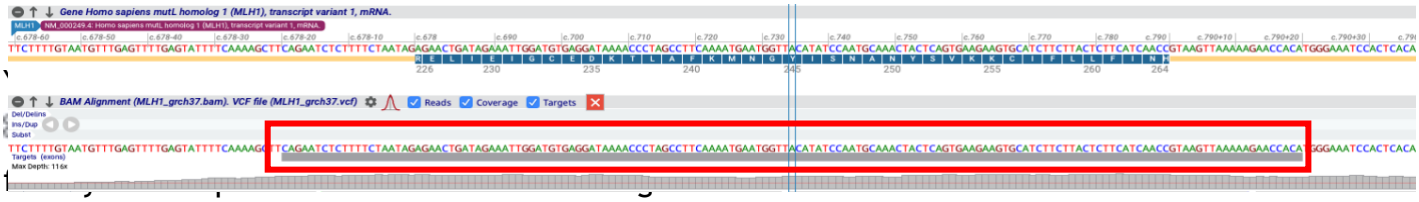
When zooming at nucleotide level the genomic reference sequence is displayed above the targets sub-track:

Hovering a read will display information related to reads as indicated in the BAM file:

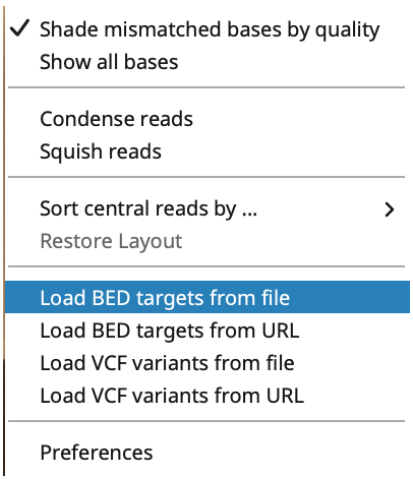


Viewing sequencing targets

In Alamut® Visual Plus, sequencing targets are supposed to cover current gene's exons with some exon-flanking intronic bases (20bp, by default):

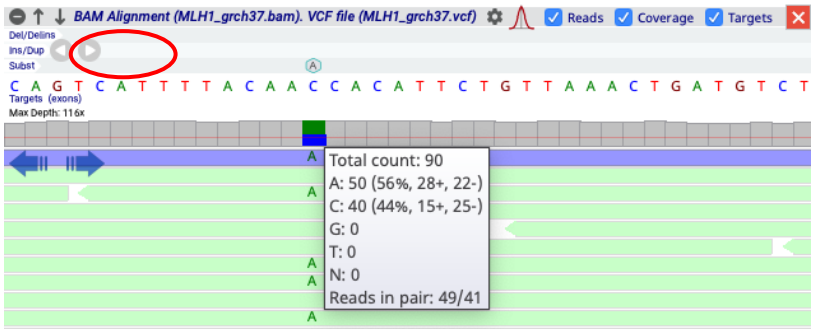


HTTP and HTTPS protocols are supported.



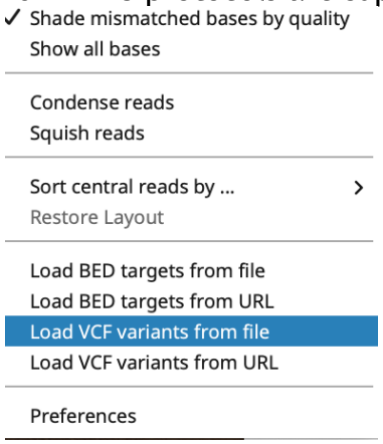
Viewing variants

Single nucleotide variants (SNVs) are automatically detected where non-reference bases are called at a frequency above the 'Allele frequency threshold' defined in the Preferences panel. Jump from one SNV to the other by clicking the arrow buttons on the left of the coverage sub-track.



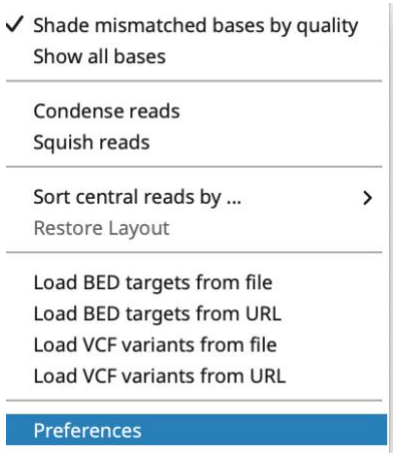
By default, the VCF is loaded when importing the BAM file if it is located in the same folder with same file name.

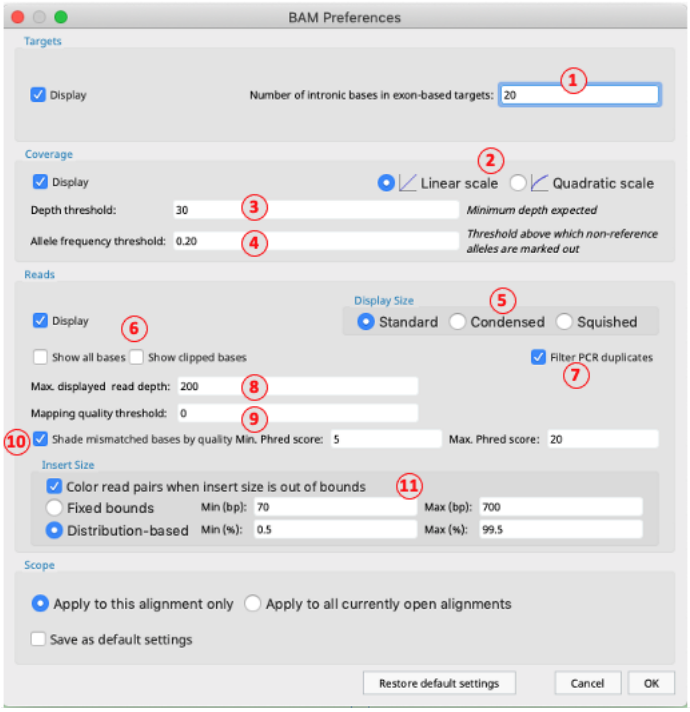
From the BAM track 'Options'  or by right clicking in the BAM track. Select "Load VCF variants from file" to load a BED file from your computer or select "Load VCF variants from URL" to load a BED file from a web server. HTTP and HTTPS protocols are supported.



Visualization options

BAM preferences are accessible through the BAM track 'Options' or by right clicking in the BAM track.





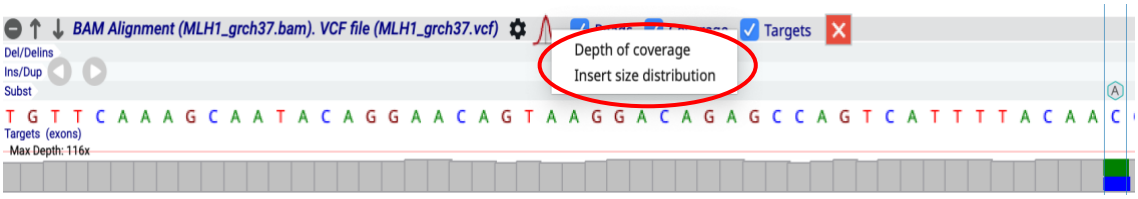
1. By default, sequencing targets are supposed to cover current gene's exons. This setting specifies the number of exon-flanking intronic bases to add to exon-defined targets.
2. The coverage histogram can either be displayed using a linear scale where the height of each bar is directly proportionate to the depth value, or using a quadratic scale where low depth values are increased and high depth values are decreased.
3. Targets are highlighted with red color where coverage depth is below this threshold. Besides, detected SNVs are only reported at positions where coverage is above this threshold.

4. Single nucleotide variants (SNVs) are detected where non-reference bases are called at a frequency above this threshold.
5. These settings define the graphical height of reads.
6. If 'Show all bases' is not checked then read bases are displayed only if they differ from the reference sequence. Some NGS data processing tools "soft-clip" bases at either end of reads if appropriate. Checking the 'Show clipped bases' option reveals these bases. This option should usually remain unchecked.
7. Alamut® Visual Plus does not itself detect PCR duplicates. Reads marked as PCR duplicates in the BAM file are withdrawn if this option is checked.
8. This setting only affects the graphical display of reads, not computations.
9. Reads with a mapping quality under this threshold are withdrawn.
10. If this option is checked then called bases with a Phred score under the specified minimum threshold are not displayed. Bases with a Phred score between the specified minimum and maximum thresholds are shaded. Bases above the maximum threshold are displayed in full color.
11. Based on insert size values provided in the BAM file, paired reads too distant or too close from each other are highlighted if this option is checked. Expected normal insert sizes can be expressed as fixed values or as a percentage over the distribution. If the insert size is large, denoting a deletion, reads are colored red. If the insert size is small, denoting an insertion, reads are colored blue.

Viewing BAM alignment statistics

Alamut® Visual Plus computes descriptive statistics from BAM file (depth of coverage and insert size). These statistics are computed for the current displayed gene locus.

Click on the picture (red histogram near options in the BAM track) and, then select either "Depth of Coverage" or "Insert Size Distribution" from the menu.

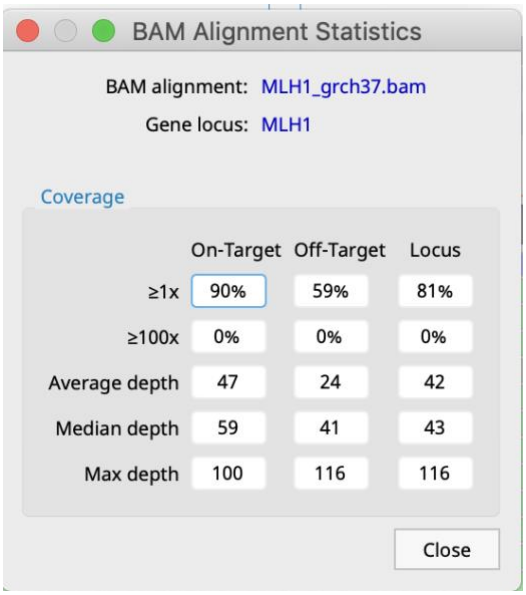


To display the depth of coverage statistics, select "Depth of Coverage" from the menu.
A table summarizes for the current gene locus, the following statistics:

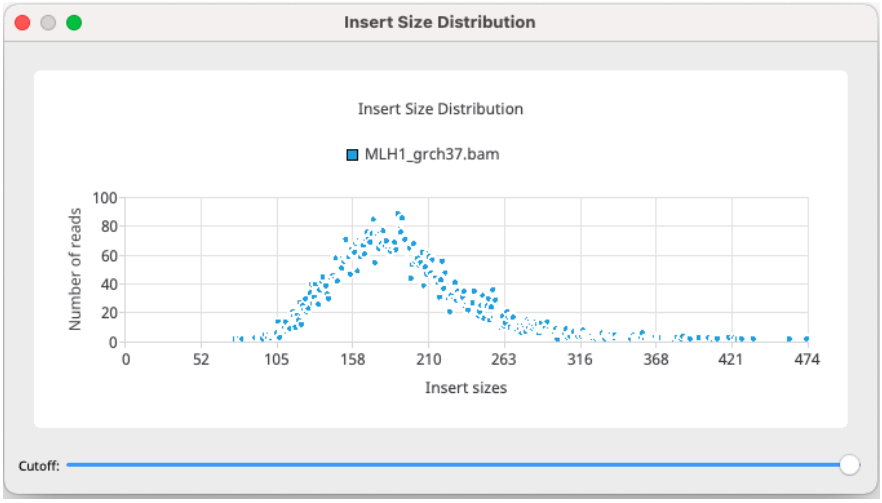
- **Depth of coverage 1X**: the percentage of a region that is covered by at least one read.
- **Depth of coverage based on a user defined coverage threshold**: the percentage of a region that is covered by at least this coverage threshold.
- **Average, Median and Maximum of the depth of coverage.**

These statistics are computed for the following regions:

- **On-Target**: targeted region (the region of interest that needs to be investigated by the sequence analysis)
  The region is based on the region defined by a user BED file or by the Alamut® Visual Plus targeted region (see 'Viewing sequencing targets' section for more details)
- **Off-Target**: region outside the targeted region (not investigated by the sequence analysis)
- **Locus**: current gene locus

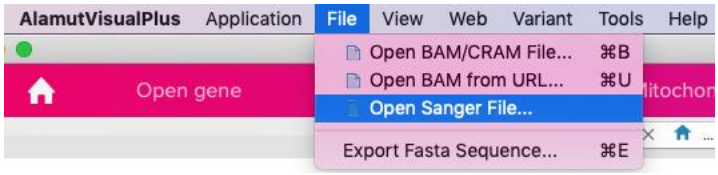


To display “Insert Size Distribution" (only for paired-end sequencing data), select "Insert Size Distribution" from the menu.
The insert sizes are computed from the reads that are in the current gene locus (displayed in Alamut® Visual Plus) and, then they are plotted as follows.
The cutoff option allows to choose an insert size interactively displayed in the graph.

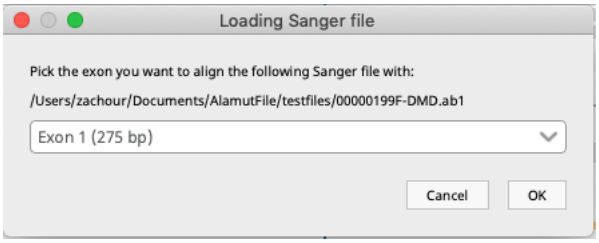


10.8.2. Viewing Sanger electropherogram

Alamut® Visual Plus allows you to upload Sanger Electropherogram (Sequencing) data by opening a new track through the “File > open Sanger file” menu.



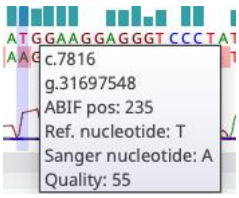
A pop-up will appear where you can select the exon where the Sanger sequencing was performed:



The Sanger track shows different components

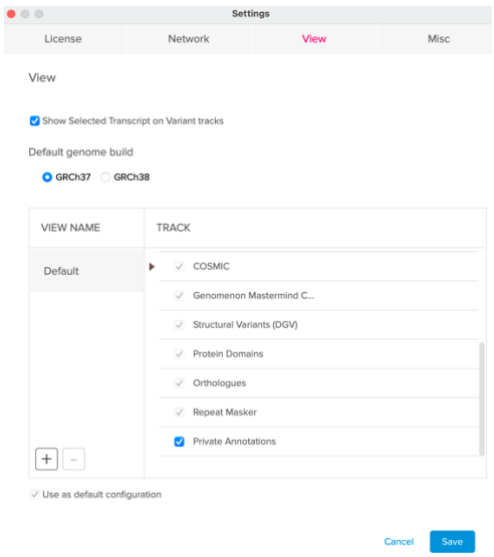
1. Quality scores displayed in bars.
2. Reference sequence. Note that when the gene is reverse, the reverse reference sequence is displayed.
3. Sanger reference. Note that when a substitution is detected, it is highlighted in red. A deletion is indicated by a '-'and highlighted in red. An insertion is indicated by an arrow.
4. Electropherogram. The display can be scaled with the 'Scale' button.
5. The quality threshold. By default, the threshold is set at 20. The user can customize the threshold. When the shows a quality score inferior or equal to the indicated 'Q-value' in the track, the bar in the quality scores sub-track is displayed in orange.
6. Print Sanger track

When hovering the nucleotide in the Sanger track, you can see the nucleotide and its coordinates as following:



10.8.3. Private Annotations

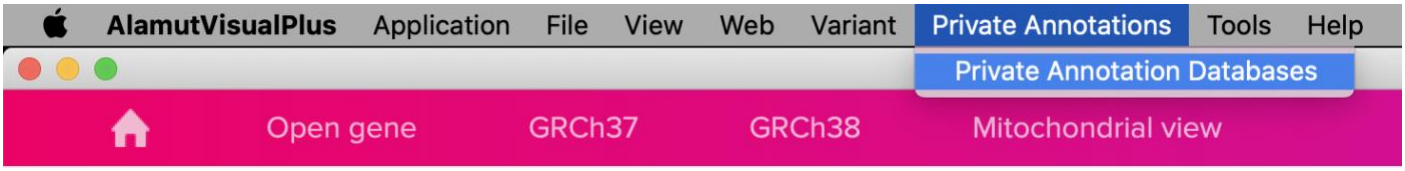
Private annotations functionality enables the user to define manually and import sequence-based annotations, such as BED files. This feature is available on a dedicated track. To switch this feature on, the corresponding track needs to be enabled from the settings View menu.



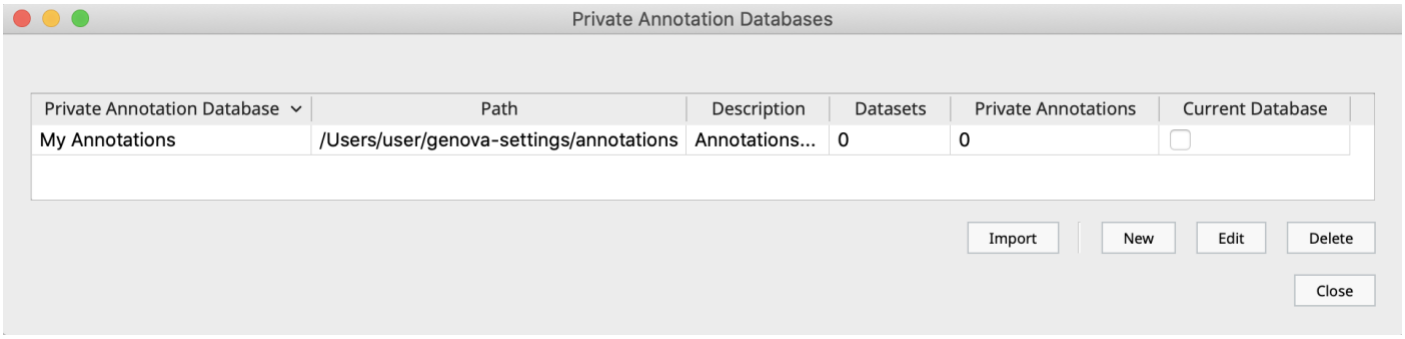
Customs Private Annotations

Creating and managing private annotation databases

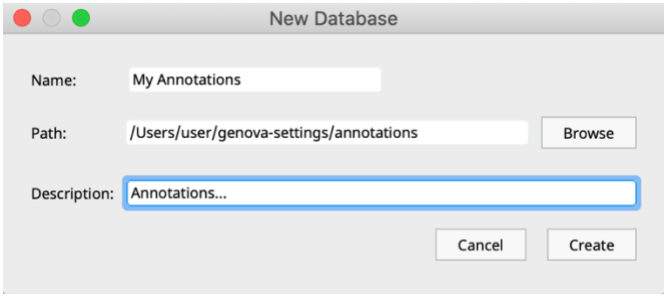
Private annotations are stored in a dedicated database. To manage your private annotation databases, click on menu > Private Annotations > Private annotation Databases.



The Private Annotation Databases window lists the existing databases. In the Private Annotations Track, you will be able to see the content of one of your Private Annotation Database. You can choose your current database by using the checkboxes in the 'Current Database' column.



To create a new database, click on 'New' button. A new pop-up will appear. You can define a new database by selecting a path, adding a name and a description.

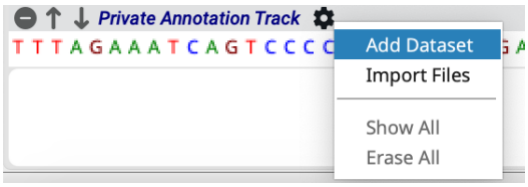


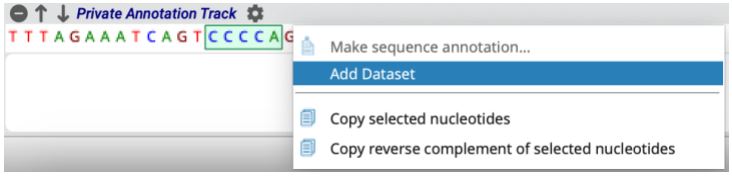
The basic private annotation database functionality implementation allows to store databases on each user's computer, or into a shared folder. When private annotations are stored in a shared file system, caution must be taken so that two people don't edit concurrently private annotations.

Creating and managing datasets

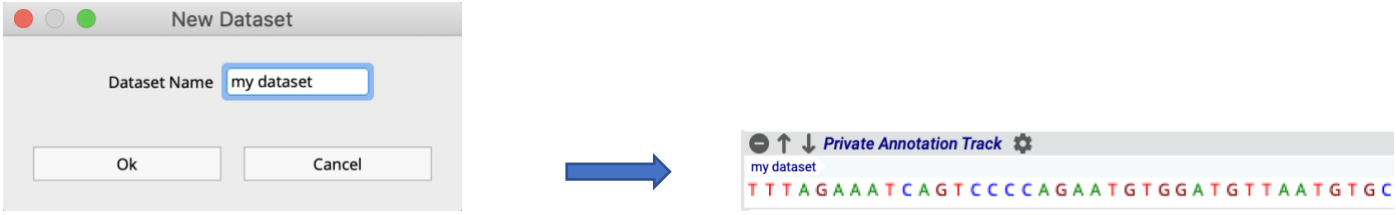
Private annotations are organized in sub-tracks called datasets. To create custom datasets, you can use the 'Options' menu ⚙ of the Private Annotation Track or the context menu of a nucleotide selection in the Private Annotation Track (by right-clicking inside the selection).

You need to have a database selected as your current private annotation database to be allowed to create custom datasets.





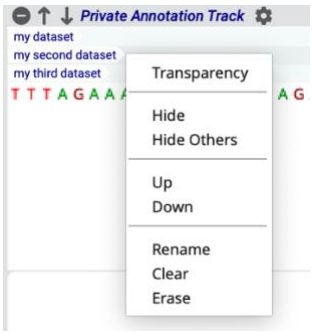
Clicking on 'Add Dataset' will open a new pop-up where you can specify the name of your new dataset. Dataset names have to be unique among a database. Clicking on 'Ok' will create the dataset and the new sub-track will appear.



Right-clicking on a dataset label will open a context menu. This context menu allows the following actions:

- **Transparency:** the private annotations are displayed with semi-transparent colors when this option is checked and with plain colors otherwise
- **Hide:** hide the dataset from the track
- **Hide Others:** hide the other datasets from the track
- **Up:** move up the dataset

- **Down:** move down the dataset
- **Rename:** rename the dataset
- **Clear:** delete all annotations of the dataset
- **Erase:** delete the dataset (from the track and from the database)

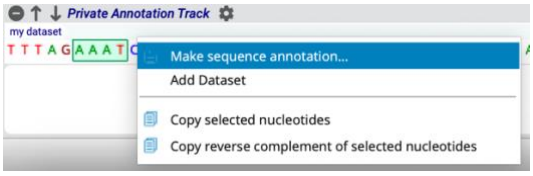


The 'Options' menu ⚙ of the Private Annotation Track contains items to manage the group of datasets: **Show All** to show all datasets when some have been hidden previously and **Erase All** to erase all the datasets.

Creating and managing Private Annotations

A new private annotation can be manually created from the context menu associated to a selection of nucleotides. The nucleotide selection can be done in the Private Annotation, the Genome or the Gene tracks.

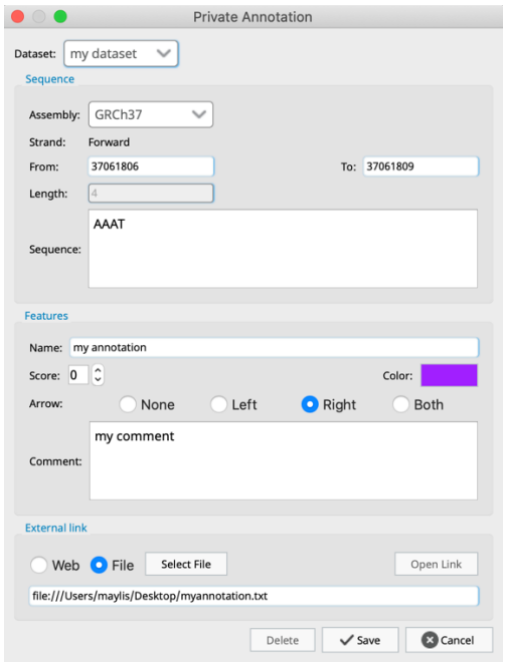
You need to have a database selected as your current private annotation database and to have at least one dataset in your current database to be allowed to create private annotations.



Clicking on 'Make sequence annotation…' will open a new pop-up where you can specify the properties of your private annotation:

- **Dataset**: the dataset/sub-track
- **Assembly:** the assembly of the annotated sequence. A private annotation is visible only on the assembly its sequence belongs to. When updating the assembly, the positions From and To will be automatically mapped to the new assembly
- **From/To:** origin and end of the annotation. The strand is automatically set according to the strand that has been used to select the nucleotides in the track. The length and the sequence are automatically computed.
- **Name:** name of the private annotation. Private annotation names have to be unique among a dataset
- **Arrow:** the arrow initialized according to the strand of the private annotation
- **Score**

- **Color**
- **Comment**
- **External link:** web link or link to an internal document

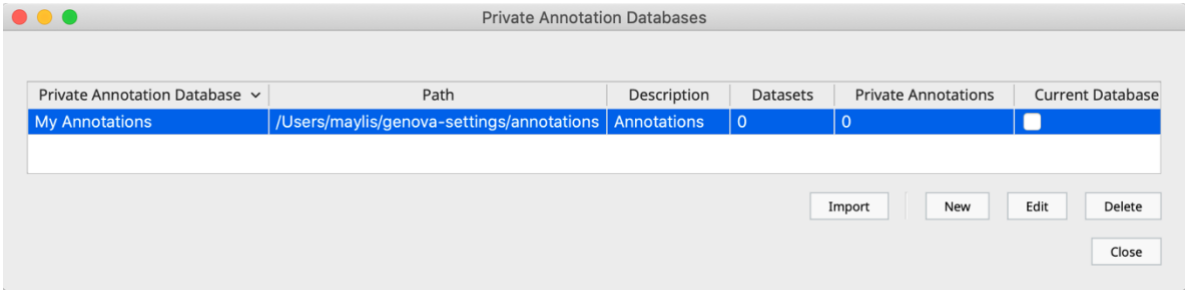


Clicking on 'Save' will create the private annotation and it will be visible in the sub-track it belongs to.

Then, to edit the private annotation information, you can either double-click on the private annotation or use its context-menu.

Import Private Annotations from Alamut Visual®

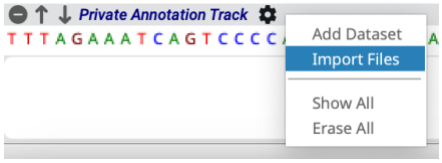
Private Annotation files from Alamut® Visual (.apa) can be imported into the Private Annotation Databases of Alamut® Visual Plus. To do so, open the Private Annotation Database window via Menu > 'Private Annotations' > 'Private Annotation Databases'. Select the database you want to import the private annotations into. Click on 'Import' and select the file you want to import.



Viewing sequence-based annotation files

Alamut® Visual Plus supports the import of annotations in BED, GFF version 2/GTF and GFF version 3 formats into the Private Annotations Track.

To import sequence-based annotation files, open the 'Options' menu ⚙ of the Private Annotation Track and click on 'Import Files'. Choose the files you want to import and validate. You can import several files at once.



Each file will generate at least one dataset and the sub-track will appear in the track. As for manually created datasets, right-clicking on a dataset label will open a context menu.

This menu contains two items to manage how the private annotations are displayed in the associated sub-track:

- **Full:** change the display in order to view all segments in full mode
- **Squish:** change the display in order to view all segments in squish mode

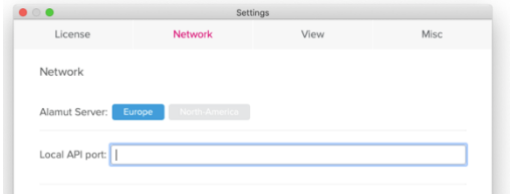
Data imported from sequence-based annotation files cannot be edited so a dataset created by an import cannot be renamed or erased.

You can visualize the information of the imported private annotations by double-clicking on it or by using its context-menu. The information of imported private annotations cannot be edited.

10.9. Alamut Visual Plus API

Alamut® Visual Plus includes a programmatic access functionality through an Application Programming Interface (API) enabling external tools to control the software. Notably, the search bar is open to external software: any software tool or web page can be customized in order to request Alamut® Visual Plus to display any information that can be processed by the search bar.

The server listens to local HTTP GET requests coming through the port that is specified in the Options dialog box (see above). The default port is set to 10000 but it can be changed to any available port in the Options dialog box (menu ‘Settings’ > ‘Network’ > ‘API’ section).



10.9.1 Specifications

Three HTTP GET requests can be processed by Alamut® Visual Plus: **version**, **search** and **open**. **search** and **open** require the user or the third-party software to provide its institution ID and API key. If you don’t have one yet, please contact support@sophiagenetics.com.

10.9.2 Version

```
http://127.0.0.1:10000/version
```

Upon receiving this request, useful for testing purposes, Alamut® Visual Plus outputs its current version and name as a json object.

10.9.3 Search

```
http://127.0.0.1:10000/search?institution=XXXXXX&apikey=YYYYYYYY&request=NM_000059.3:c.4563A>C
```

This request asks Alamut® Visual Plus to display any data available in the system (here variant “NM_000059.3:c.4563A>C”). Any request that can be processed by the search field of Alamut® Visual Plus (see **10.4.3. Search bar (Extended Access Feature))** can be processed with the “search” verb.

10.9.4 Open

```
http://127.0.0.1:10000/open?institution=XXXXXX&apikey=YYYYYYYY&filetype=bam&path=http%3A%2F%2Frd-connect.interactive-biosoftware.com%2FBAM%2Fexample.bam
```

This request asks Alamut® Visual Plus to open a BAM file located at `http://rd-connect.interactive-biosoftware.com/BAM/example.bam` (the path is percent encoded, see https://www.urlencoder.org). A gene or a genomic region has to be opened beforehand in this case via the **search** verb.
The path to a local path should contain file:// as in the example below:

```
http://127.0.0.1:10000/open?institution=XXXXXX&apikey=YYYYYYYY&filetype=bam&path=file%3A%2F%2F%2FUsers%2Ftoto%2FMLH1%20grch37.bam
```

This will open `file:///Users/toto/MLH1 grch37.bam`.

The **open** API call supports BAM, CRAM and Sanger file types.

A detailed description of the API can be found here:
https://extranet.interactive-biosoftware.com/alamut-visual-plus_API.html

10.10. Miscellaneous

Menus

- Application
  - Open Gene
  - GRh37
  - GRh38
  - Mitochondrial view
  - Home
- File
  - Open BAM/CRAM File
  - Open BAM from URL
  - Open Sanger File
  - Export Fasta Sequence
- View
  - Focus on
  - Show ruler
  - Color nucleotides
  - Use amino acid 3 letter code
  - Full Screen
  - Show navigation bar
  - Increase Font
  - Decrease Font
  - Reset Font

    - Enter Full Screen
- Web
    - View gene in Ensembl Browser
    - View displayed region in Ensembl Browser
    - View entire region in NCBI Sequence Viewer
    - View entire region in UCSC Browser
    - View HGNC symbol report for this gene
    - View gene in OMIM® web site
    - View gene in GENATLAS web site
    - View gene in Gene Reviews web site
    - View Uniprot entry for the product of this gene
- Variant
    - New Variant
    - Local Variants Database
- Tools
    - Genetic Code
    - Compare Amino Acids
    - Assembly Mapping
    - Take Screenshot
    - Nomenclature validation dialog
    - Variant validation dialog
- Help
    - Software Documentation
    - Data Sources
    - License Agreement
    - Software Reference
    - Contact Support

Navigation with keyboard
Use the following keys to navigate in Alamut® Visual Plus:

- **Left arrow key**: step shift the sequence to the left
- **Right arrow key**: step shift the sequence to the right
- **Up arrow key**: step shift the tracks upwards
- **Down arrow key**: step shift the tracks downwards

11. Quality Control Procedures

External genomic data has been successfully tested and implemented in Alamut database.
Frontend functionalities and data display have been successfully tested and implemented

12. Other Information

12.1. Training

Before using the Software, video tutorials are provided in the software homepage to get started.
Further requests on live demo and support can be addressed to :
support@sophiagenetics.com

12.2. Responsibility

This system solely provides assistance to the intended user and does not substitute or replace the intended user's experience and/or responsibility during its use. It must always be possible for the user to proceed without the assistance of the system.

12.3. Documentation

This user manual describes the use of an interpretation software for genomic variations that must be used with care. It is therefore important that all users of the software:

- Read this guide carefully before use
- Have access to this guide at all times

13. Symbols

User Manual, Operating instructions

Read User Manual, Read Operating instructions

Research Use Only

Reference Number

Manufacturer

14. Support

In case of difficulty using the product, contact our support line and e-mail mentioned on the "Summary Information" page of this user guide.