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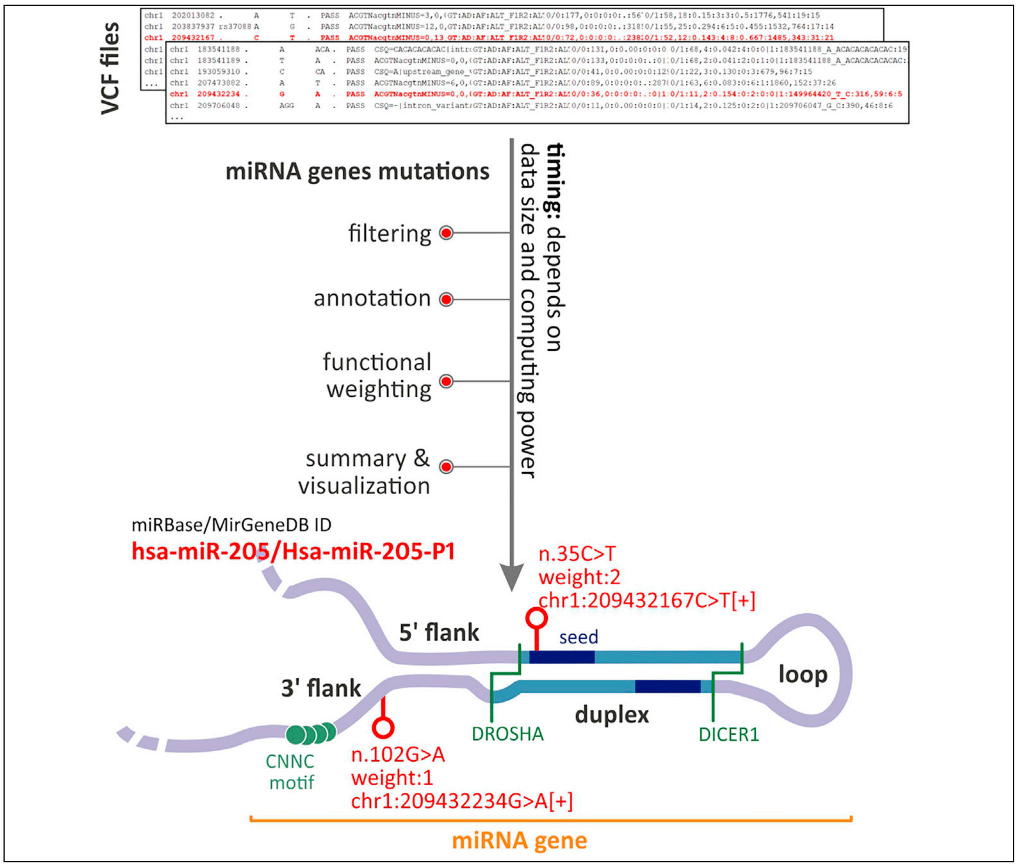
Protocol

miRMut: Annotation of mutations in miRNA genes from human whole-exome or whole-genome sequencing



```
chr1  202013082 .        A      T  .  PASS  ACGTNacgtnMINUS=3,0,(GT:AD:AF:ALT_F1R2:AL0/0:177,0:0:0:0:.:56 0/1:58,18:0.15:3:3:0.5:1776,541:19:15
chr1  203837937 rs37088 A      G  .  PASS  ACGTNacgtnMINUS=12,0,GT:AD:AF:ALT_F1R2:AL0/0:98,0:0:0:0:.:318!0/1:55,25:0.294:6:5:0.455:1532,764:17:14
chr1  209432167 .        C      T  .  PASS  ACGTNacgtnMINUS=0,13 GT:AD:AF:ALT_F1R2:AL0/0:72,0:0:0:0:.:238:0/1:52,12:0.143:4:8:0.667:1485,343:31:21
chr1
chr1
chr1
...
```

```
chr1  183541188 .    A    ACA .  PASS  CSQ=CACACACACAC|intr(GT:AD:AF:ALT_F1R2:AL0/0:131,0:0.00:0:0:0 0/1:68,4:0.042:4:0:0|1:183541188_A_ACACACACACAC:19
chr1  183541189 .    T    A   .  PASS  ACGTNacgtnMINUS=0,0,(GT:AD:AF:ALT_F1R2:AL0/0:133,0:0:0:0:.:0|:0/1:68,2:0.041:2:0:1:0|1:183541188_A_ACACACACACAC:
chr1  193059310 .    C    CA  .  PASS  CSQ=A|upstream_gene_(GT:AD:AF:ALT_F1R2:AL0/0:41,0:0.00:0:0:12!0/1:22,3:0.130:0:3:679,96:7:15
chr1  207473882 .    A    T   .  PASS  ACGTNacgtnMINUS=6,0,(GT:AD:AF:ALT_F1R2:AL0/0:89,0:0:0:0:.:287(0/1:63,6:0.083:0:6:1:1860,152:37:26
chr1  209432234 .    G    A   .  PASS  ACGTNacgtnMINUS=0,0,(GT:AD:AF:ALT_F1R2:AL0/0:36,0:0:0:0:.:0|1:0/1:11,2:0.154:0:2:0:0|1:149964420_T_C:316,59:6:5
chr1  209706048 .    AGG  A   .  PASS  CSQ=-|intron_variant(GT:AD:AF:ALT_F1R2:AL0/0:11,0:0.00:0:0:0|:0/1:14,2:0.125:0:2:0|1:209706047_G_C:390,46:8:6
...
```

Here, we present the miRMut protocol to annotate mutations found in miRNA genes based on whole-exome sequencing (WES) or whole-genome sequencing (WGS) results. The pipeline assigns mutation characteristics, including miRNA gene IDs (miRBase and MirGeneDB), mutation localization within the miRNA precursor structure, potential RNA-binding motif disruption, the ascription of mutation according to Human Genome Variation Society (HGVS) nomenclature, and miRNA gene characteristics, such as miRNA gene confidence and miRNA arm balance. The pipeline includes creating tabular and graphical summaries.

Martyna O. Urbanek-Trzeciak, Piotr Kozlowski, Paulina Galka-Marciniak

murbanek@ibch.poznan.pl (M.O.U.-T.)
pgalka@ibch.poznan.pl (P.G.-M.)

Highlights

miRMut is a software to annotate mutations in miRNA genes

miRMut utilizes VCF or CSV files generated based on WGS or WES results

miRMut assigns different mutation characteristics and potential functional impact

The output is presented in tabular and graphical summaries

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Protocol

miRMut: Annotation of mutations in miRNA genes from human whole-exome or whole-genome sequencing

Martyna O. Urbanek-Trzeciak,[1,2,*] Piotr Kozlowski,[1] and Paulina Galka-Marciniak[1,3,*]

[1]Department of Molecular Genetics, Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznan, Poland

[2]Technical contact

[3]Lead contact

*Correspondence: murbanek@ibch.poznan.pl (M.O.U.-T.), pgalka@ibch.poznan.pl (P.G.-M.)

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SUMMARY

Here, we present the miRMut protocol to annotate mutations found in miRNA genes based on whole-exome sequencing (WES) or whole-genome sequencing (WGS) results. The pipeline assigns mutation characteristics, including miRNA gene IDs (miRBase and MirGeneDB), mutation localization within the miRNA precursor structure, potential RNA-binding motif disruption, the ascription of mutation according to Human Genome Variation Society (HGVS) nomenclature, and miRNA gene characteristics, such as miRNA gene confidence and miRNA arm balance. The pipeline includes creating tabular and graphical summaries.
For complete details on the use and execution of this protocol, please refer to Urbanek-Trzeciak et al. (2020).

BEFORE YOU BEGIN

Most genetic studies and, therefore, developed software have focused on the protein-coding part of the genome. In recent years, noncoding variants found in promoters, untranslated regions (UTRs), introns, or microRNAs have also started to be recognized as potential disease drivers (Elliott and Larsson, 2021; Rheinbay et al., 2020; Tan, 2020). The importance of mutations found in the noncoding portion of the genome suggests that new tools for annotation need to be developed. miRNA gene mutation annotation may be used for (i) prioritization of variants detected in miRNA genes and (ii) analysis of functional variant enrichment, e.g., for identification of positive selection signals or identification of cancer-driving genes as it is commonly used in protein-coding sequences (based on the ratio of missense to synonymous mutations ascertained to be more functional and neutral, respectively). There are numerous tools for annotation of coding variants (e.g., ANNOVAR (Annotate Variation), VEP (Ensembl Variant Effect Predictor), SIFT (Sorting Intolerant From Tolerant), and PMUT (Predicting pathological MUtations)) (Ejigu and Jung, 2020; Pabinger et al., 2014; Shameer et al., 2016), but miRMut is, to our knowledge, the first annotation tool for mutations in miRNA genes.

Originally, the pipeline was used utilizing compressed variant call format (VCF.GZ) files from The Cancer Genome Atlas (TCGA) project containing somatic mutation data from >10,000 cancer samples from 33 cancer types (Urbanek-Trzeciak et al., 2020) (project ID: 16565; phs000178.v11.p8). As miRNA genes, we defined pre-miRNA-coding sequences extended upstream and downstream by 25 nucleotides. The pre-miRNA-coding sequences were reconstructed as described previously (Galka-Marciniak et al., 2019).

Note: The user should already have performed WES/WGS and created a variant call format (VCF) or VCF.GZ files with a list of the identified variants/mutations, which serve as the main input files of the protocol. The input files may also contain mutations outside of miRNA genes, which are filtered out in subsequent steps of the procedure. The user may also use files generated previously in any external project. The generation of VCF/VCF.GZ files based on unmapped sequence reads can be performed with the use of the genome analysis toolkit (GATK) developed by the Broad Institute (Cibulskis et al., 2013; Garcia et al., 2020; Van der Auwera, 2020). Alternatively, the user may use a comma-separated values (CSV) file containing mutations as described in more detail in the section Adding miRNA-specific information to specific mutations.

Note: The scripts assume that all needed quality filtering was done before, while creating VCF/VCF.GZ files. If multiple sequencings were performed or multiple files were generated for a single sample (as occurs in some TCGA somatic mutation files), they should be combined, or the issue should be resolved otherwise to avoid distortion. An example solution for TCGA file merging is described in the troubleshooting section under problem 1.

Reference files

Reference files for human miRNA genes (hg38 and hg19) are available at the GitHub repository and within supplementary files, and we recommend using these files. Reference files include information on miRNA gene coordinates (**coordinates_{genome version}.bed**), miRNA characteristics (arm balance and high confidence miRNAs), miRNA gene subregions [flanking sequence 5′ (flanking-5), duplex before seed (pre-seed), seed (seed), duplex after seed (post-seed), loop, and flanking sequence 3′ (flanking-3)], and gene orientation (**localizations_{genome version}.csv**) as well as miRBase-based precursor sequences (**coordinates_with_seq_{genome version}.bed**).

Coordinates_{genome version}.bed file includes coordinates of miRNA genes in the following structure:

chr1	17343	17456	hsa-mir-6859–1
chr1	30384	30483	hsa-mir-1302–2
chr1	187865	187978	hsa-mir-6859–2

Each coordinate is in a new line and has a tab separator between the following columns: chromosome, start, stop, and miRNA_gene_ID. No header should be present in the file. Start and stop coordinates are both inclusive.

Coordinates_with_seq_{genome version}.bed file has a structure as **coordinates_{genome version}.bed** file with an additional column (last) with the sequences of the genomic regions. The file is necessary for the prediction of disruptions of sequence motifs caused by the mutations.

Localizations_{genome version}.csv file is a CSV file with columns as follows (for a more precise column description, please see Tables 2 and S1): chrom (chromosome, e.g., chr1), name (name of miRNA region, e.g., hsa-mir-6859–1_flanking-3), ID (miRBase miRNA ID, e.g., MI0022705), start (first position (inclusive) of region, e.g., 17344), stop (last position (inclusive) of region, e.g., 17368), orientation (miRNA gene orientation, e.g., -), based_on_coordinates (information, if the region coordinates were calculated based on miRBase coordinates or only one mature miRNA was defined in miRBase and coordinates must have been predicted by the script, e.g., yes), arm (side of miRNA precursor (or loop) in which mutation will be located, i.e., 5p, 3p, or loop), type (region type, i.e., flanking sequence 5′ (flanking-5), duplex before seed (pre-seed), seed (seed), duplex after seed (post-seed), loop, and flanking sequence 3′ (flanking-3)), pre_name (miRBase pre-miRNA name, i.e., hsa-mir-6859-1), start_pre_build (miRBase start coordinate), stop_pre_build (miRBase stop coordinate), confidence (as high-confidence miRNA genes, we considered genes of miRNA precursors annotated as "high confidence" in miRBase and/or deposited in MirGeneDB v2.0), balance (according to the number of reads reported for the particular pre-miRNA arm (miRBase), the analyzed precursors were classified into one of 3 categories as follows: (i) generating mature miRNA predominantly from the 5p arm ($\geq$90% of reads from the 5p arm) - 5p; (ii) generating mature miRNA predominantly from the 3p arm ($\geq$90% of reads from the 3p arm) - 3p; and (iii) balanced (>10% of reads from each arm) - both; if no reads were available, the balance was assigned as unknown), mirgenedb_ID (ID from MirGeneDB if available, i.e., Hsa-Mir-8-P2a_pre)

Note: In the reference files, we excluded miRNAs (n = 9) that were problematic in the context of the second strand and, therefore, reliable structure prediction (hsa-mir-4489, hsa-mir-4539, hsa-mir-1469, hsa-mir-657, hsa-mir-4325, hsa-mir-548bb, hsa-mir-4285, hsa-mir-548o, and hsa-mir-601). All of the excluded miRNAs had low confidence according to miRBase and were not annotated in the MirGeneDB database.

Note: The reference files were primarily prepared based on miRBase v.22.1 (Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2019) and MirGeneDB v2.0 (Fromm et al., 2020) for the human genome (hg38). Hg19 files (**coordinates_hg19.bed**, **coordinates_with_seq_hg19.bed**, and **localizations_hg19.csv**) were prepared as liftover from hg38 files, and some of the miRNAs (n=8) were removed during liftover (hsa-mir-6859–2, hsa-mir-10401 (3 genomic coordinates), hsa-mir-486–1, hsa-mir-486–2, hsa-mir-1234, hsa-mir-4477b, hsa-mir-4477a, and hsa-mir-532). For other releases and/or other organisms, please refer to the troubleshooting section under problem 2.

Installing prerequisites

Please refer to the wiki pages of GitHub repository for more details on prerequisites and installation. In particular, additional options of setup using Docker (see also problem 3) or using conda environments (see also problem 5) are presented there.

1. Downloading or cloning GitHub repository
   a. Clone GitHub: https://github.com/martynaut/mirnome-mutations repository, e.g., through ssh protocol using the command in a terminal:

```
>git clone git@github.com:martynaut/mirnome-mutations.git
```

The repository can be also downloaded as a zip file and unpacked in a chosen directory.

A copy of the repository (release version v1.0.1, Zenodo: https://doi.org/10.5281/zenodo.5574501) is available in Data S1 (however, the users are strongly encouraged to use GitHub version of the software, where the newest version will be available).

2. Installing Python prerequisites
   a. The user is encouraged to use a virtual environment for the project to avoid Python library conflicts among projects, but it is not a requirement. Example steps for conda virtual environment are shown in the troubleshooting section under problem 5.
   b. Change working directory to the repository folder:

```
>cd mirnome-mutations
```

   c. Install Python prerequisites from **requirements.txt** file. The scripts were primarily tested and are recommended to run using Python 3.8. Run command:

```
>pip install -r requirements.txt
```

Note: The scripts were tested on Ubuntu (20.04.3 LTS) and MacOS (Big Sur) with both Python 3.7 and Python 3.8 clean environments; therefore, in the commands presented in the manuscript, we use Unix notation of paths. Due to the use of specific Python libraries (e.g., hgvs), it is not currently straightforward to use the miRMut pipeline on Windows.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
TCGA data	Campbell et al. (2020)	https://gdc.cancer.gov/
miRBase	Kozomara and Griffiths-Jones (2014) and Kozomara et al. (2019)	http://www.mirbase.org/index.shtml
MirGeneDB	Fromm et al. (2015, 2020)	https://mirgenedb.org/
Software and algorithms		
ViennaRNA	Lorenz et al. (2011)	https://www.tbi.univie.ac.at/RNA/#download
miRMut code	GitHub	GitHub: https://github.com/martynaut/mirnome-mutations
miRMut Docker image	Docker Hub	https://hub.docker.com/repository/docker/martynaut/mirmut

STEP-BY-STEP METHOD DETAILS

We prepared a GitHub repository containing all the necessary reference files, scripts, and example inputs and outputs for the method presented below. The repository wiki pages contain information on installation and running miRMut. The repository is found at

GitHub: https://github.com/martynaut/mirnome-mutations.

To follow the step-by-step instructions, users are encouraged to download the whole repository, including the example input files. For instructions on how to clone the repository and install the required Python libraries, please refer to the "installing prerequisites" section in "before you begin".

It is expected that users will run the entire process all at once, but it is also possible to run the steps independently (1 - filtering miRNA gene mutations, 2 – processing mutation list, 3 - adding miRNA-specific information to specific mutations, 4 - calculating mutation weights, 5 - generating mutation CSV summaries, and 6 - generating mutation visualizations). Here, we describe how to run the script all at once, and later how to run each step separately. For default to run the whole procedure, the user should run the following command within the repository:

```
>python3 run_mirnaome_analysis.py \

/path/to/input/folder/with/vcfs \

/path/to/output/folder/ \

/path/to/reference/files/coordinates.bed \

/path/to/reference/files/localizations.csv \

/path/to/reference/files/coordinates_with_seq.bed
```

To run the script on example files provided in the repository, run the following command:

```
>python3 run_mirnaome_analysis.py \ input_files/input_vcf/example_vcf_set \

output_folder/user_example_output_from_vcf \
reference_files/coordinates_hg38.bed \
reference_files/localizations_hg38.csv \
reference_files/coordinates_with_seq_hg38.bed
```

The already calculated output for this command can be found within the repository in the output_folder/example_output_from_vcf folder.

Non-default values for all optional arguments used and described in each of the six steps can also be defined for a full run as follows (for parameters, please refer to Table 1):

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-m 1 -f 1
```

If the script is run but not completed, the user may define from which step analysis should start using the "from_step" ("-s") optional argument, e.g.,

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 2
```

The command will start the analysis from step 2 of the procedure.

The end step of the analysis can also be defined (e.g., if the full analysis is not yet wanted) using the "end_step" ("-es") optional argument as follows:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 2 \
-es 2
```

Table 1. All command line parameters for the miRMut scripts

Parameter	Function	Scripts that accepts the parameter
–from_step, -s	Accepts a number value from 1 to 6. Starts analysis from chosen step (inclusive), where: 1 – Filtering miRNA gene mutations 2 – Processing mutation list 3 – Adding miRNA-specific information to specific mutations 4 – Calculating mutation weights and HGVS nomenclature 5 – Generating summaries 6 – Generating visualization of mutations Note that previous steps need to be run before as each step depends on output files from earlier steps. Note that –from_step parameter is overwritten when –csv_file parameter is used if defined –from_step value is lower than 3.	run_mirnaome_analysis.py
–end_step, -es	Accepts a number value from 1 to 6. Stops the analysis on the chosen step (inclusive). For numbers description see –from_step parameter.	run_mirnaome_analysis.py
–include_merger, -m	Accepts values 0 (disabled) or 1 (enabled). It must be enabled if analysed VCF/VCF.GZ files were created with multiple algorithms (e.g., MuSE, MuTect2, etc.). If the merger of algorithms is enabled, mutations are grouped by position, individual ID of patient, and mutation type (reference and alternative allele), and if the same mutation is found in files generated by different algorithms, it is treated as one mutation. This transformation prevents counting the same mutation multiple times. If the merger of algorithms is enabled, the "indiv_name" has to be defined in the VCF/VCF.GZ files.	run_mirnaome_analysis.py, merge_algorithms.py
–include_filtering, -f	Accepts values 0 (disabled) or 1 (enabled). If filtering is enabled, for each "results_{file type}.csv" file, "results_{file type}_eval.csv" is created with an additional column "eval", which contains information if the mutation passes filtering as defined below. If filtering is enabled, the following criteria are taken into account (only if available in VCF/VCF.GZ files): • SSC > 30 • BQ of an alternative allele in tumor sample > 20 • QSS of an alternative allele in tumor samples divided by alternative allele count in tumor samples > 20 • At least two mutation-supporting reads in a tumor sample (if no mutation-supporting read was detected in the corresponding normal sample) OR at least 5 × higher frequency of mutation-supporting reads in the tumor sample than in the corresponding normal sample Please note that some of the filtering criteria are strictly algorithm-related and are not relevant in other projects.	run_mirnaome_analysis.py, merge_algorithms.py
–csv_file, -c	Accepts path to a CSV file. The parameter is used when the miRMut is to be run on a CSV file instead of VCF/VCF.GZ files. The CSV file needs to be formatted as defined in adding miRNA-specific information to specific mutations section. When used with run_mirnaome_analysis.py script, the input folder argument will not be used (but needs to be defined), and the script will automatically start from step 3 (unless a later step is defined using –from_step parameter), skipping the first two steps.	run_mirnaome_analysis.py, add_mirna_info.py
–pass_arg, -p	Accepts values 0 (disabled) or 1 (enabled). When PASS filtering is enabled using –pass_arg parameter only mutations with PASS value in the FILTER column from VCF/VCF.GZ files are included in the analysis. Although there is this option, it is recommended to do VCF/VCF.GZ filtering prior to the miRMut usage.	run_mirnaome_analysis.py, extract_results_for_mirnaome.py
–weight_filter, -w	Accepts values 1, 1.5 and 2. The default value is set to 1. It filters out only mutations with selected weight or higher. It is done on the 4th step of the script and affects files with hgvs nomenclature, summaries and figures.	run_mirnaome_analysis.py, add_weghts.py

To run analyses of the example files, the reusable bash file with Python commands (**run_test.sh**, available in the repository) may be used. It can be executed by running the following command in a terminal:

```
>./run_test.sh
```

Note: The TCGA analysis in Urbanek-Trzeciak et al. (2020) was run with -m, -f and -p flags. For details, please refer to the original manuscript. The descriptions of the parameters can be found in Table 1.

Note: For 1500 VCF.GZ files (1.61GB) on a PC with Ubuntu 20.04.3 LTS (8GB RAM; 3.20GHz CPU) it took about 2h40 to process complete miRMut analysis (step 1 took approx. 1h40, step 2 – 20s, step 3 – 5s, step 4 – 10min, step 5 – 1s, step 6 – 50min).

Filtering miRNA gene mutations

Timing: hours to days; factors that affect timing include the number and size of samples as well as available computing resources.

Within the first step from the provided VCF and/or compressed VCF.GZ files, mutations within miRNA gene coordinates are filtered and gathered in a CSV file. This is usually the longest step of the procedure. To run this step, the **coordinates_{genome version}.bed** reference file, which is described in the "before you begin" section, is required.

Additionally, the user needs to provide a path to the folder with VCF/VCF.GZ files (all files may be in a single folder but subfolders are allowed) and a path to the folder where output files should be saved (if the folder does not exist, it will be created; and if it exists, the current content of the folder will be removed).

1. To run only the first step of the script, use one of the following commands:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 1\
-es 1
```

or

```
>python3 extract_results_for_mirnaome.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed
```

There are several output files saved in the defined output folder from this part of the protocol: **files_-summary.csv**, which summarizes files included in the analysis, depending on information included in the VCF/VCF.GZ files, it includes individual/sample names and IDs, tumor/normal sample names and IDs, and types of algorithms used for mutation detection; **results_{file type}.csv**, file is created for each file type, where "file type" is an algorithm name used for VCF file creation (if the information is available in the VCF file), e.g., MuTect or MuSE.

Note: If all VCF files are created with a single algorithm, only one results file will be created. If multiple algorithms are used (e.g., 4 algorithms in TCGA data), multiple files will be created. In the second step of the procedure, the user may choose to merge mutations identified by different algorithms.

Note: In the pipeline, it is assumed that all needed filtering has been completed during VCF/VCF.GZ files preparation. However, during this step, it is possible to filter only mutations that passed all filters (has FILTER assigned to PASS value), which can be achieved by using the optional argument "-p" as follows:

```
>python3 extract_results_for_mirnaome.py \

/path/to/input/folder/with/vcfs \

/path/to/output/folder/ \

/path/to/calculated/input/files/coordinates.bed \

-p 1
```

As we encourage users to filter VCF files prior to use of miRMut software, by default, the filtering of only PASS variants is disabled.

Note: If no VCF/VCF.GZ files are found in the input directory, the pipeline will be terminated.

IMPORTANT: Please note that if the defined output folder does exist, the current content of the folder will be removed by the script.

Processing mutation list

Timing: minutes to hours; factors that affect timing include the number of found mutations and available computing resources.

The second step of the pipeline involves processing the mutation list, including the following two optional transformations: a merger of mutations identified by different algorithms (if more than one algorithm is used for mutation detection); and additional internal filtering of mutations as defined below. The optional transformations are disabled on default, but they may be enabled using two flags ("-m" and "-f") as shown below. The flags might be used in a similar way when running the whole pipeline at once.

If the merger of algorithms is enabled, mutations are grouped by position, individual ID of patient, and mutation type (reference and alternative allele), and if the same mutation is found by different algorithms, it is treated as one mutation. This transformation prevents counting the same mutation multiple times.

If additional internal filtering is enabled, the following criteria are taken into account (only if available in VCF/VCF.GZ files): SSC (Somatic Score) > 30 - parameter determined by VarScan2 and SomaticSniper; BQ (Base Quality) of an alternative allele in tumor sample > 20 - parameter determined by MuSE; QSS (Sum of base quality scores for each allele) of an alternative allele in tumor samples divided by alternative allele count in tumor samples > 20 - parameter determined by MuTect2, at least two mutation-supporting reads in a tumor sample (if no mutation-supporting read was detected in the corresponding normal sample) OR at least 5 × higher frequency of mutation-supporting reads in the tumor sample than in the corresponding normal sample.

Please note that some of the filtering criteria are strictly algorithm-related and are not relevant in other projects.

2. To run this step of the script, use one of the following proposed commands enabling needed options:
   a. Second step without merging and filtering

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed\
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 2 \
-es 2
```

or

```
>python3 merge_algorithms.py /path/to/output/folder/
```

   b. Second step of the script enabling both merger of various algorithms and filtering of mutations

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 2 \
-es 2 \
-m 1 \
-f 1
```

or

```
>python3 merge_algorithms.py /path/to/output/folder/ \
-m 1 \
-f 1
```

This step generates multiple files. If filtering is enabled, for each **results_{file type}.csv** file, **results_{file type}_eval.csv** is created with an additional column "eval", which contains information if the mutation passes filtering as defined above. Additionally, **all_mutations.csv** file is created, including list of mutations described by chromosome (chrom), nucleotide position (pos), reference allele (ref), alternative allele (alt), and if available, individual name (indiv_name), reference allele count in normal sample (norm_ref_count), alternative allele count in normal sample (norm_alt_count), reference allele count in tumor sample (tumor_ref_count) alternative allele count in tumor sample (tumor_alt_count), and algorithm used (alg).

Note: If VCF/VCF.GZ files were created with multiple algorithms but the merger of algorithms is not enabled, the analysis will be terminated on this step with a relevant message. Similarly, if no mutations in miRNA genes were found in defined input files, the script will be terminated.

IMPORTANT: If the merger of algorithms is enabled, the ''indiv_name'' has to be defined in the VCF/VCF.GZ files.

Adding miRNA-specific information to specific mutations

Timing: seconds to minutes; factors that affect timing include the number of mutations and available computing resources.

During this step, miRNA mutations listed in the ''**all_mutations.csv**'' file generated in the previous steps are annotated. The annotation is based mainly on the information gathered in the ''**localizations_{genome version}.csv**'' files. The following pieces of information are annotated based on the mutation position: miRNA ID (from miRBase and MirGeneDB), miRNA name, miRNA region (flanking sequence, loop, and mature miRNA), miRNA confidence (information if miRNA found in miRBase is believed to be true miRNA), and balance (5′miRNA or 3′miRNA depending on the main miRNA generated from a particular miRNA gene based on miRBase data). Additionally, mutation type is defined (substitution/insertion/deletion) if relevant, and substitution type is defined if relevant.

Alternatively, the user may run this part of the script (and following steps) on an external CSV file. The CSV file needs to contain the following columns: chrom (with chromosome defined as, e.g., chr1), pos (with nucleotide position of a mutation in the chromosome), ref (reference allele), alt (alternative allele), and indiv_name (with sample ID; the script will accept empty values here but to enable all summaries sample ID is recommended). To execute the script on a CSV file, please see step 3 (last option). The **example_csv.csv** example CSV file is provided in the repository in the input_files/input_csv folder.

3. To run this step of the script, use one of the following commands:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 3 \
-es 3
```

or

```
>python3 add_mirna_info.py /path/to/output/folder/ \
/path/to/reference/files/localizations.csv
```

Alternatively, this step may be run on any CSV file fulfilling the requirements defined earlier, using option "-c":

```
>python3 add_mirna_info.py /path/to/output/folder/ \
/path/to/reference/files/localizations.csv \
-c /path/to/csv/file/with/mutations.csv
```

This step generates the **all_mutations_with_localization.csv** file, which includes a list of mutations based on the **all_mutations.csv** file (or user-defined file with mutations) with annotated information.

Note: It is possible to run the whole script at once on a CSV file using the "-c" optional argument as follows:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-c /path/to/csv/file/with/mutations.csv
```

In this case, the input folder argument will not be used (but needs to be defined), and the script will automatically start from step 3, skipping the first two steps.

Calculating mutation weights and HGVS nomenclature

During the fourth step, the potential functional impact of each mutation is evaluated based on the mutation characteristics and location. A mutation receives a higher score (weight) if it is located within the DROSHA or DICER1 (key endonucleases involved in miRNA biogenesis) cleavage site, is located within a miRNA duplex, especially in the seed region, and is located in binding sites of proteins known to play role in miRNA biogenesis.

The scoring is as follows: seed region mutation - weight 2; cleavage sites, duplex (excluding seed region) or disturbance of protein binding motif - weight 1.5; all other mutations - weight 1.

Additionally, during this step, mutation designation according to the HGVS nomenclature is defined. Both genomic (using chromosomal position) and noncoding DNA coordinate (using miRBase pre-miRNA coordinate) designations are calculated.

4. To run this step of the script, use one of the following commands:

```
>python3 run_mirnaome_analysis.py \ /path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 4 \
-es 4
```

or

```
>python3 add_weights.py \
/path/to/output/folder/ \
/path/to/reference/files/coordinates_with_seq.bed
```

This step generates the three files: **all_mutations_with_weights.csv**, which includes a list of mutations with additional information on motifs and weights; **all_mutations_with_hgvs.csv**, which includes all information as the previous file and genomic HGVS designation; **all_mutations_with_n_hgvs.csv**, which includes all information as the previous file and miRBase HGVS designation.

Note: If a mutation is predicted to disturb more than one functional site, the highest weight is counted.

Note: Sequence motifs analyzed within the script can be found in the **add_weights_helpers.py** file in the repository. The sequence motif calculations were based on previously published scripts (Urbanek-Trzeciak et al., 2018).

Generating summaries

Timing: seconds to minutes; factors that affect timing include the number of mutations and available computing resources.

In this step, files summarizing mutations in miRNA genes are generated, including summaries from the miRNA gene perspective, specific mutation perspective, and complex mutation (explained below) perspective.

5. To run this step of the script, use one of the following commands:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 5 \
-es 5
```

or

```
>python3 distinct_occur.py \
/path/to/output/folder/
```

This step generates the three files: **occur.csv** file, which reports the number of mutations found in each miRNA gene in the analyzed cohort, for each miRNA gene (characterized by chromosome, pre-miRNA name, and miRNA miRBase ID), the count of unique samples with at least one mutation (indiv_name_nunique), count of all mutations (indiv_name_count), and count of unique mutation positions (pos_nunique) are provided, additionally, complex mutations, if identified in a particular miRNA, are reported; **distinct_mutations.csv** file, which summarizes distinct mutations (genome positions with specific alteration), including the number of patients/samples with the mutation, if available, the sum of reads supporting reference and alternative alleles are also provided; **complex_mutations.csv** file, which includes information on complex mutations defined as multiple nucleotide alterations found in a single miRNA gene in a single sample/patient (please note: if the "indiv_name" (sample identifier) is not defined, this file will be inutile as all mutations will be treated as found within a single sample). The file shows a number of mutations (pos_count) and unique mutation positions (pos_nunique) found in each miRNA gene (characterized by chromosome, pre-miRNA name, and miRNA miRBase ID) in each sample (indiv_name) and if any of the mutations are complex.

IMPORTANT: Do not use **complex_mutations.csv** if the individual name (indiv_name) is not defined (empty or nonunique) in the VCF/VCF.GZ or CSV files. In such a case, the "if_complex" column in the **occur.csv** file is also inutile.

Generating visualization of mutations

Timing: minutes to hours; factors that affect timing include the number of mutations in miRNA genes and available computing resources.

During this step, figures graphically presenting mutations on a schematic structure of miRNA genes are generated. For visualization of mutations and to enable examination of sequence variants in the context of miRNA precursor structures, we superimposed the identified variants on the consensus miRNA precursor structure and categorized them according to localization in the miRNA gene subregions. Generated figures depict (i) all found variants (Figure 1A), (ii) variants found in miRNA genes with the dominant 3′ miRNA, (iii) variants found in miRNA genes with the dominant 5′ miRNA, and (iv) balanced miRNAs. Similar figures are generated separately for each miRNA gene with at least one mutation in the provided dataset (example shown in Figure 1B). All figures are generated as Scalable Vector Graphics (SVG) files to enable further editing in graphic software.

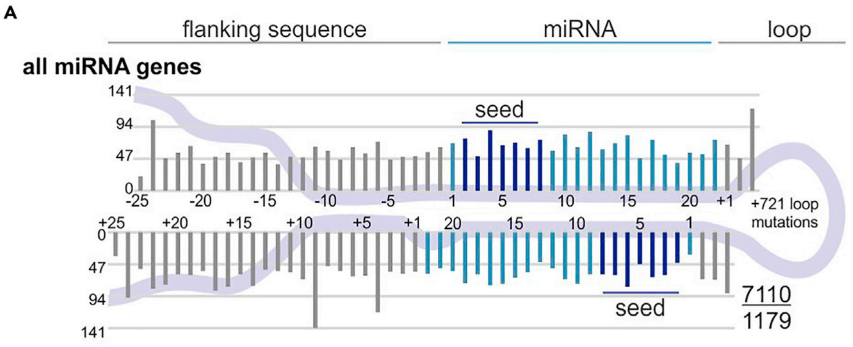
6. To run the sixth step of the script, use one of the following commands:

```
>python3 run_mirnaome_analysis.py \
/path/to/input/folder/with/vcfs \
/path/to/output/folder/ \
/path/to/reference/files/coordinates.bed \
/path/to/reference/files/localizations.csv \
/path/to/reference/files/coordinates_with_seq.bed \
-s 6 \
-es 6
```

or

```
>python3 mutation_loc_figures.py \
/path/to/output/folder/
```

A



B

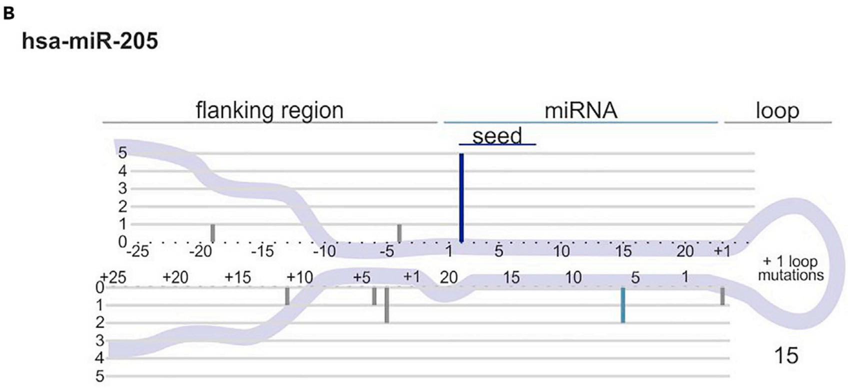


Figure 1. Localization of mutations detected by the script
(A) All mutations found in the pan-cancer cohort in TCGA (Urbanek-Trzeciak et al., 2020).
(B) Mutations found within miR-205 in the pan-cancer cohort in TCGA (Urbanek-Trzeciak et al., 2020). miRNA duplex positions are indicated in blue, and seed regions are indicated in dark blue. The flanking regions and terminal positions of the apical loop are indicated in gray. The numbers in the lower right corner represent the number of plotted mutations (upper) and the number of mutated miRNA genes (lower; not provided for mutations shown within a single miRNA gene). If present, sequence variants localized beyond position 22 in longer mature miRNAs are cumulatively shown at position 22. The plot shows mutations within six positions of the loop (first 3 and last 3 nucleotides). The number of the remaining loop mutations is indicated within the loop.

EXPECTED OUTCOMES

Steps 1–5 from the step-by-step method generate CSV files. The output files are briefly described in each step, and example outputs are provided in the GitHub repository in the "output_folder" folder. A description of each column found in all CSV files is included in Tables 2 and S1.

Step 6 generates figures. Figure 1 shows example output figures, which partially resembles Figures 2 and S3 in our companion paper by Urbanek-Trzeciak et al. (2020). Example figures may also be found in the "output_folder" in the GitHub repository.

LIMITATIONS

The protocol was tested on several VCFs and VCF.GZ files generated with different pipelines; however, taking into account the diversity of this file type, some files may not be feasible. In such a case, the solution may be the conversion of the data to the CSV format (as described above in step 3 - adding miRNA-specific information to specific mutations section.

With increasing knowledge of miRNA biogenesis and function, including verified miRNA-target interactions, the assessment of mutation functional impact may be further developed.

Table 2. Mutation list column description in alphabetical order.

Column name	Column description	Possible values
alg	algorithm used for detection of mutations	e.g.,: mutect2, somatic sniper
alt	alternative allele	e.g.,: G, A, GAA
arm	in which part of miRNA precursor mutation is located	5p, loop, 3p
balance	arm from which the main miRNA is generated; defined based on deep sequencing reads (miRBase), [for details see Urbanek-Trzeciak et al. (2020)]	5p, 3p, both, undefined
based_on_coordinates	indicates whether used coordinates were defined based on miRBase annotations or were predicted based on miRNA structure	yes, no
chrom	chromosome	e.g.,: chr1, chrX
confidence	as high-confidence miRNA genes, we considered genes coding for miRNA precursors annotated as "high confidence" in miRBase and/or deposited in MirGeneDB v2.0	High, Low
cut_region	whether the mutation is within Drosha or Dicer cut region	0 (no), 1 (yes)
duplex	whether the mutation is within duplex	0 (no), 1 (yes)
from_start	nucleotide position from start of miRNA region in which mutation is located (e.g., within the seed, 5′ flanking sequence)	e.g.,: 1, 3
from_end	nucleotide position from end of miRNA region in which mutation is located (e.g., within the seed, 5′ flanking sequence)	e.g.,: -1, -25
hgvs	noncoding HGVS nomenclature of the mutation in the reference to the miRBase coordinates *Note that annotation of some indels may not match the latest HGVS recommendations; for multimutations (multiple changes in a single position in one sample) HGVS nomenclature is not available	e.g.,: n.90G>A, n.67+21G>A, n.1–15delA
hgvs_g	genomic HGVS nomenclature of the mutation; for multimutations HGVS is not available	e.g.,: NC_000010.11:g.17845137C>A, NC_000001.11:g.1167872_1167873insT
ID	miRBase ID	e.g.,: MI0000737
indiv_name	sample identifier	e.g.,: sample01
mirgenedb_ID	mirGeneDB ID	e.g.,: Hsa-Mir-8-P1a_pre
motifs	whether any motif was disturbed as a result of the mutation	0 (no), 1 (yes)
motifs_{}	information for each checked motif if there was the loss of a motif as a result of the mutation	-, loss
mutation_type	mutation type: substitution, insertion, deletion, insertion-deletion, multimutation	subst, ins, del, indel, multimutation
name	miRNA region name built by pre-miRNA name and precursor region connected by an underscore	e.g.,: hsa-mir-200a_flanking-5, hsa-mir-511_post-seed
no_of_loc	internal check column	1
orientation	miRNA gene orientation	+, -
pos	genomic position of the mutation	e.g.,: 1167859, 20633667
pre_name	precursor name	e.g.,: hsa-mir-200a, hsa-mir-6084
ref	reference allele	e.g.,: C, GCA
seed	whether mutation is located within seed	0 (no), 1 (yes)
start	miRNA region start coordinate	e.g.,: 1167853, 20633667
start_pre_build	miRBase start coordinate	e.g.,: 1167863, 20633679
stop	miRNA region stop coordinate	e.g.,: 1167877, 20633691
stop_pre_build	miRBase stop coordinate	e.g.,: 1167952, 20633788
type	region of miRNA precursor	flanking-5, pre-seed, seed, post-seed, loop, flanking-3
type_of_subst	substitution type, n.a. for non-substitution mutations	transition, transversion, n.a.
weight	weight score	1, 1.5, 2

A description of all the columns present in transitional or summary files is included in Table S1.

miRMut was tested and is supported on Ubuntu (tested on Ubuntu 20.04.3 LTS) and MacOS (tested on Big Sur) systems.

TROUBLESHOOTING

Problem 1

In some projects, multiple sequencing is performed on a single sample, e.g., TCGA project (see before you begin). Treating such samples separately is not recommended as some mutations specific for the sample (patient or tumor) may be redundant and, in some cases, generate artifacts in downstream analyses. However, in unique situations, e.g., if sections of the same cancer sample are tested in the context of cancer heterogeneity, the particular sections may need to be considered separate samples.

Potential solution

Here, we provide a script that merges VCF.GZ files. The script was prepared to be compatible with VCF.GZ files provided by TCGA consortium and may not be suitable for other files.

The script checks all the VCF.GZ files gathered in the input directory, defines which samples have multiple files available, merges the files, saves the files in the output folder, and moves all unique VCF.GZ files to the output folder. Alternatively, the user may force the script to create a copy of the input files (instead of moving them), keeping the original files intact in the input directory.

This step assumes that the user cloned the repository of the project as described in the previous sections.

To run the script, use one of the following commands:

```
>python3 merge_vcf_files.py \
/path/to/input/folder/with/vcfs \
/path/to/new/output/folder/
```

If the user wants to copy unchanged files instead of moving them, the command is as follows with parameter -c defined:

```
>python3 merge_vcf_files.py \
/path/to/input/folder/with/vcfs \
/path/to/new/output/folder/ \
-c 1
```

There are several output files in addition to merged and copied/moved VCF.GZ files saved in the defined output folder: **files_summary_before_merging.csv** - summary of files in the defined input directory before merger, including the path to the files, sample identifier (indiv_name, indiv_ID, sample_ID_tumor_name, sample_ID_tumor_aliQ, sample_ID_normal_name, sample_ID_normal_aliQ), and algorithm used to create VCF.GZ file (type_of_file); **files_summary_count_per_patient_before_merging.csv** - count of files per sample using the same algorithm; **not_unique_patients.csv** – a fragment of the **files_summary_count_per_patient_before_merging.csv** file with nonunique samples; **do_not_use.txt** - list of paths to files of nonunique samples that are used to create merged files.

IMPORTANT: Please note that if the defined output folder does exist, the current content of the folder will be removed by the script.

Problem 2

The VCF/VCF.GZ files were prepared on a different genome version for the human genome or for another organism (see reference files section).

Potential solution

Reference files may be prepared based on the reference files available within the repository and described in the "reference files" section for any organism and genome version. Alternatively, we provide a script that creates reference files based on files from miRBase and MirGeneDB. The GitHub repository needs to be cloned as defined in the "installing prerequisites" section.

To install ViennaRNA, there are two options. Option 1 is to install from source code, in which case you should download ViennaRNA distribution from https://www.tbi.univie.ac.at/RNA/#download to a selected folder and install ViennaRNA using the following instructions:

```
>tar -zxvf ViennaRNA-2.4.18.tar.gz

>cd ViennaRNA-2.4.18

>./configure

>make

>sudo make install
```

Option 2 is to install by pip. Within your Python environment, run:

```
>pip install viennarna
```

After installing ViennaRNA, you need to prepare input files from miRBase and MirGeneDB. From miRBase, download the following files (from ftp site for chosen miRBase release https://www.mirbase.org/ftp.shtml): **hairpin.fa**, **hsa.gff3.txt**, **confidence.txt**, **confidence_score.txt**, **aliases.txt**, and **mirna_chromosome_build.txt**, From MirGeneDB, download the following file (https://mirgenedb.org/download): **hsa.gff** (Genomic coordinates gff file).

Run **prepare_localization_file.py** script in a similar way as shown below on the downloaded files:

```
>python3 prepare_localization_file.py \

/path/to/ViennaRNA-2.4.11 \

/path/to/hairpin.fa \

/path/to/hsa.gff3.txt \

/path/to/confidence.txt \

/path/to/confidence_score.txt \

/path/to/aliases.txt \

/path/to/mirna_chromosome_build.txt \

/path/to/hsa.gff \

/path/to/output/folder
```

Note: One may also use the Docker image where the Vienna package is already pre-installed (for details see problem 3).

Problem 3

Running into dependency error while installing Python packages from **requirements.txt** (see installing prerequisites section).

Potential solution

When using conda (Python environment management system) for virtual environment handling, the user may encounter dependency error if the base Python has libraries depending on CPython installed before. It is caused by how conda is handling the base space and the fact that the HGVS library depends on CPython. For more details please refer to the conda GitHub issues (GitHub: https://github.com/conda/conda/issues/7173). The possible solution would be to use virtualenv instead of conda for virtual environment handling as virtualenv does not have this issue. However, acknowledging that conda has several advantages over virtualenv it is also possible to use Python 3.7 instead of 3.8 (if Python 3.8 is the user's base Python version) for miRMut.

Alternatively, the user can use the provided Docker image. The latest Docker image can be found in the Dockerhub: https://hub.docker.com/repository/docker/martynaut/mirmut. The Docker image contains Python 3.8 with all required Python libraries installed, including ViennaRNA. We recommend using **docker-compose.yml** file to run the Docker image to automatically copy the output files to the host location. Example **docker-compose.yml** file is available within the miRMut GitHub repository.

Using the **docker-compose.yml** file, the Docker image can be run using a command:

```
>docker-compose run app
```

Problem 4

Low quality mutation calls (see before you begin section).

Potential solution

Our protocol starts with the list of already called variants (VCF files) and it is assumed that all needed filtering has been completed during VCF/VCF.GZ files preparation. But for the reliability of the analysis, it is worth paying attention to the quality of the sequencing, and reliability of mutation calls affected among others by the depth of coverage. This should be noted that the coverage below 10× or 30× in the case of sequencing of normal and tumor samples, respectively, may not be sufficient and many variants may not be properly identified or missed (Xiao et al., 2021). To filter out mutations that are likely false positives users may use –pass_arg and –include_filtering parameters in the miRMut pipeline. When PASS filtering is enabled only mutations with PASS value in the FILTER column from VCF/VCF.GZ files are included in the analysis. Similarly, when the additional internal filtering is enabled (which includes the parameters described in step 2 "processing mutation list"), only mutations that fulfill the criteria are included in the analysis. For details please refer to the flag descriptions in Table 1.

Problem 5

The user has a different Python version installed than recommended in the manuscript (see installing prerequisites section).

Potential solution

The scripts were tested on Python 3.7 and 3.8, it is not recommended to use earlier versions and Python 3.9 as not all libraries may work in the environment. If the user is using a different version of Python by default, we recommend using conda for clean environments with specific Python versions.

To create a clean environment with required libraries follow the instructions:

1. Download and install conda (for details please see https://conda.io/projects/conda/en/latest/user-guide/install/index.html)

2. Create a new conda environment with Python 3.8:

```
>conda create -name mirmut_env python=3.8
```

3. Activate virtual environment

```
>conda activate mirmut_env
```

4. Install all needed libraries from requirements file (working directory needs to be the repository folder)

```
>pip install -r requirements.txt
```

5. Perform all needed analyses using miRMut

6. After working with miRMut deactivate virtual environment

```
>conda deactivate
```

Next time using miRMut, the user only needs to activate the environment (step 3), run analyses (step 5), and deactivate the environment (step 6).

For more detail on using conda virtual environments see: https://conda.io/projects/conda/en/latest/user-guide/getting-started.html#managing-environments.

Alternatively, use the prepared Docker image with miRMut software with all installed dependencies. For the details, please see the problem 3 section.

If the user encounters any problem not addressed in the troubleshooting section, please create an issue in the miRMut GitHub repository.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Paulina Galka-Marciniak (pgalka@ibch.poznan.pl).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The code is available at GitHub repository: GitHub: https://github.com/martynaut/mirnome-mutations. Example input and output files are provided. To rerun full TCGA-based pan-cancer analysis as shown in Urbanek-Trzeciak et al. (2020), a request to TCGA consortium needs to be made.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.101023.

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AUTHOR CONTRIBUTIONS

Protocol Design and Conceptualization – P.K., P.G-M., and M.O.U-T.; Pipeline Execution and Documentation - M.O.U-T; Pipeline testing P.G-M. and M.O.U-T.; Manuscript Writing - Review and Editing - P.G-M., M.O.U-T., and P.K.; Funding Acquisition and Supervision - P.K., P.G-M., and M.O.U-T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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