LEXOGEN

Enabling complete transcriptome sequencing

Accurate Analysis of RNA-Seq Data

RNA-Seq data analysis software

User Guide

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1. Introduction

This manual describes the system requirements and the license activation process of the Mix^2 software. In addition, command line options of the software are discussed as well as its input and output format. For further questions related to the Mix^2 software please contact bioinfo@lexogen.com.

2. Requirements

The Mix² software runs on Linux x64 distributions. The graphical user interface of the Mix² license manager requires GTK+ 2.6 or higher and the official PNG reference libraries (libpng 12.0). During operation the software will access port number 36963[1] , which therefore has to be free. There is no data flow via this port. It is used only for the synchronization of multiple running instances of the software.

To run the Mix² software on a computer cluster, please contact us at bioinfo@lexogen.com.

The Mix² software has been tested on:

- Ubuntu 12.04+ Desktop x64
- Ubuntu 12.04 Server x64
- openSUSE 13.2 Desktop x64
- openSUSE 12 Server x64
- Linux Mint 17.1 Desktop x64
- Fedora Live 20 Desktop x64
- CentOS 7.0 Desktop x64

If you encounter any problems when running the Mix² software, please contact us at bioinfo@lexogen.com.

[1]This port is normally used for "Counter Strike".

3. Mix² License Manager

Prior to running the Mix² software a license needs to be downloaded and activated via the Mix² license manager. If the license is to be deployed in a global directory to allow access for multiple users, then the license manager must be run with root privileges.

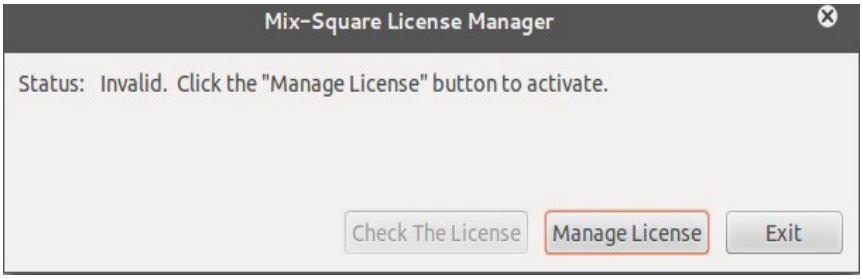

Figure 1. License Manager Main Window

Figure 1 shows the main window of the license manager. Clicking on the "Check The License" button will check the validity of the license. The license can be downloaded and activated by clicking on the "Manage License" button.

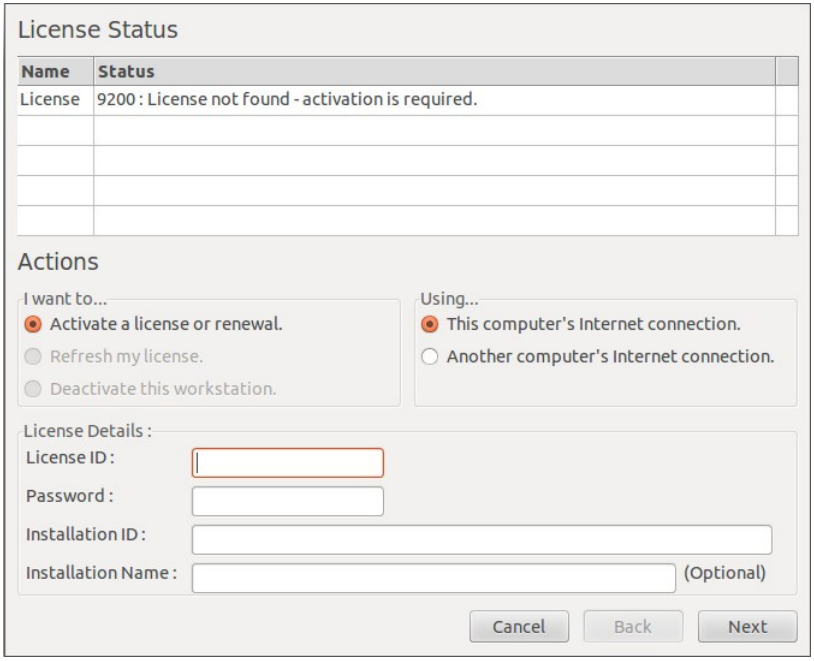

Figure 2. License Activation Window

Figure 2 shows the license activation window, which appears after clicking the "Manage License" button in the main window of the license manager (Figure 1). The license management window provides information regarding the license status and can be used to complete the license activation process. A number of actions are defined in this window.

- Activate a license or renewal: This option is used to activate and download a license using the License ID and Password obtained for a trial version or through purchase of the software. Upon the activation request an Installation ID will be assigned to the system on which the license is activated. Providing an installation name serves the purpose of making licenses easily distinguishable and is optional.
- Refresh my license: This option is used to refresh a license status. If a license is extended, the software will usually download the new license file automatically upon checking for the license status. However, if the software fails to refresh the license automatically the "Refresh my license" option can be used to manually request a license refresh.
- Deactivate this workstation: This option allows to deactivate a license on a workstation and to activate this license instead on another workstation. The number of reactivations on different workstations is limited depending on the type of license.
- This computer's internet connection: This option activates and downloads the license using the internet connection of the computer on which the license manager is running.
- Another computer's Internet connection: If the computer, on which the license is to be installed, does not have Internet connection, then the license can be downloaded through another computer's connection. The option "Another computer's Internet connection" option is used to generate the XML license request file. The XML request file then needs to be uploaded to the license server manual response page (https://secure.lexogen.com/solo/customers/ManualRequest.aspx) and an XML response file has to be downloaded. The latter is then used to create the license file for the computer without Internet connection.

If the Mix^2 software is to be run on a computer without window manager, e.g. a typical server, the graphical user interface of the Mix^2 license manager can be exported to another machine with window manager. This is achieved by logging into the computer without window manager from the computer with window manager with ssh using the –X switch and subsequent execution of the license manager on the remote machine.

4. Running Mix²

The Mix² software can be run from the command line as follows:
./mix-square [options] <arguments>

Options

General Options:	
-h [--help]	Describe options.
-G [--GTF] arg	Directory of the reference annotation file. Please refer at Mix ² Input section.
-B [--BAM] arg	Directory of the RNA-Seq read alignments in BAM format. SAM file format is not supported. The alignments need to be sorted by their leftmost coordinates.
-o [--output-dir] arg	Sets the output directory which the results will be saved to. The default is a directory called "output" in the current working directory. If the path to output-dir is relative it will be generated within the current working directory.
-p [--threads] arg	Number of threads to be used for the estimation process. The max number of threads can be used depends on the license type.
Advanced Abundance Estimation Options:	
-x [--max-total-frags] arg	Sets the maximum number of fragments in a locus. A locus which has more fragments than the maximum number is skipped. Genes skipped can be found in genes_skipped.list. Default: 5000000
-M [--max-comp-frags] arg	Sets the maximum number of valid fragments in a locus. A locus which has more valid fragments than the maximum number is skipped. Genes skipped can be found in genes_skipped.list. Default: 5000000
-m [--min-comp-frags] arg	Sets the minimum number of valid fragments in a locus. A locus with less valid fragments than the minimum number is skipped. Genes skipped can be found in genes_skipped.list. Default: 1
-q [--min-param-diff] arg	Sets the minimum parameter difference between 2 iterations. Default: 1e-5
-i [--nr-iterations] arg	If the minimum Log Likelihood condition is not reached then the EM algorithm will terminate if the maximum number of iterations is reached. Default: 500
-T [--likelihood-threshold] arg	Sets the minimum log likelihood difference between 2 iterations. If the log likelihood difference between two iterations is below this value, then the EM algorithm terminates. Default: 0.5
-L [--genes-list] arg	A file containing gene IDs which are included or excluded in the experiment.
-b [--blocks] arg	This number defines how many mixture components are used to model the bias of fragment startsites. This number can be understood as the 'resolution' of the pdf. Accepted values are natural numbers from 1 to 10. The default is 3.
-e [--exclude-genes]	With this option, mix-square model excludes the genes which are specified in the genes list file via the –L option.

-t [--global-tying]	With this option, global tying is turned on which means that all the isoforms of a gene share the same parameters for the fragment start distributions This options should only be used if the relative fragment start distributions of the isoforms within a gene can be expected to have a similar shape, or in case of data sparsity.
-l [--log-files]	Turns on estimation process logging. An individual file is created for each gene.
Advanced Program Behavior Options:	
-s [--license-status]	With this option, you can view some information related to your license.
-r [--ignore]	With this option, the warnings, which may be shown while using the max-frags-locus option, are turned off.
-d [--debug]	This option turns on the debugging mode. This should only be used to obtain diagnostic information when facing problems with mix-square.

5. Mix² Input

GTF (gene transfer) format and a file which contains the alignments in BAM (binary SAM) format.

The structure of the annotation file should be like:
<seqname> <feature> <start> <end> <strand> [attributes]

Field number	Field name	Example	Description
1	seqname	19	The name of the sequence. Chromosome ID or contig ID.
2	feature	Exon	Record type which can be "CDS", "start codon", "stop codon", "intron", "exon", "transcript" etc. All the record types are ignored except "exon".
3	start	51456206	Start coordinate of the feature, in this case the start coordinate of the exon.
4	end	51456321	End coordinate of the feature, in this case the end coordinate of the exon.
5	strand	+	The strand which exon comes from. Should be "-" or "+".

Attribute number	Attribute name	Example	Description
1	gene_id	ENSG00000167754	A globally unique identifier for the genomic locus of the transcript.
2	transcript_id	ENST00000391809	A globally unique identifier for the transcript.
3	gene_name	KLK5	The name of the gene.
4	end	51456321	End coordinate of the feature, in this case the end coordinate of the exon.

If one of the above fields/attributes is missing, the entry is skipped.

If an experiment needed to be done on a specific list of genes, then -L option could be used. That option expects a file which includes the gene IDs (one gene ID per line). A typical list should be as below:

ENSG00000167754
ENSG00000187999
ENSG00000123437
ENSG00000145310

Optionally, the -e flag can be used to exclude the genes specified in the genes-list.

6. Mix² Output

6.1. BAM Index File

Mix² will produce an index file for the input BAM file if no such index file is present.

6.2. Genes_summary file

Field number	Field name	Example	Description
1	gene_ID	ENSG00000167754	A globally unique identifier for the genomic locus of the transcript.
2	gene_name	KLK5	The name of the gene.
3	locus	19:51446559-51456349	The locus which the gene is referenced to. Chromosome ID:start coordinate - end coordinate.
4	frags_locus	20000	Number of fragments in the specified locus.
5	frags_expt	200000000	Total number of fragments in the experiment.
6	FPKM_THN	452420.36	FPKM total hits norm. FPKM_THN is calculated counting all fragments including those which are not compatible with any reference transcript. FPKM_THN is calculated continuously during the experiment.
7	comp_frags_locus	10000	Number of fragments in the specified locus, which are compatible with a reference transcript. comp_frags_locus should be used to calculate isoform row counts for differential expression analysis. comp_frags_locus should be used to calculate isoform row counts for differential expression analysis.
8	comp_frags_expt	100000000	Total number of fragments in the experiment, which are compatible with a reference transcript.
9	FPKM_CHN	904840.73	FPKM compatible hits norm. FPKM_CHN is calculated counting only the fragments, which are compatible with a reference transcript. FPKM_CHN is calculated at the end of the experiment. FPKM_CHN should be used for differential expression analysis.
10	status	OK	Whether the estimation process was successful or not.

6.3. Transcripts_summary file

Field number	Field name	Example	Description
1	tracking_ID	ENST00000391809	A unique identifier for the transcript.
2	gene_ID	ENSG00000167754	A globally unique identifier for the genomic locus of the transcript.
3	gene_name	KLK5	The name of the gene.
4	locus	19:51446559-51456349	The locus which the gene is referenced to. Chromosome ID:start coordinate - end coordinate.
5	length	1405	Transcript length in basepairs.
6	fragment_validity_coverage	0.93	Validity coverage for the specified transcript.
7	abundance	0.23416	Estimated relative abundance.
8	frags_locus	20000	Number of fragments in the specified locus.
9	frags_expt	200000000	Total number of fragments in the experiment.
10	FPKM_THN	452420.36	FPKM total hits norm. FPKM_THN is calculated counting all fragments including those, which are not compatible with any reference transcript. FPKM_THN is calculated continuously during the experiment.
11	comp_frags_locus	10000	Number of fragments in the specified locus, which are compatible with a reference transcript.
12	comp_frags_expt	100000000	Total number of fragments in the experiment, which are compatible with a reference transcript.
13	FPKM_CHN	904840.73	FPKM compatible hits norm. FPKM_CHN is calculated counting only the fragments, which are compatible with a reference transcript. FPKM_CHN is calculated at the end of the experiment.

6.4. Error_log file

This file contains information about the genes skipped because of problems during the estimation process.

6.5. Genes_skipped file

This file contains information about genes skipped due to the configuration of Mix^2, e.g. genes with more than the allowed maximum number of valid reads.

7. Test Case

The distribution of the Mix^2 software contains a small test set of artificial data, which enables the user to try out the basic functionality of the software. The example directory contains a GTF file for gene KLK5 and a sorted BAM file.

Here are two examples for how Mix^2 can be run from the command line on the test data:

- ./mix-square -G example/KLK5.gtf -B example/KLK5.sorted.bam
  - In order to run Mix^2 the above parameters are required at least. Since no output directory is specified, the results are saved in the current working directory under a directory called output.

- ./mix-square -G example/KLK5.gtf -B example/KLK5.sorted.bam -b 3 -t -o test-example-data
  - 'In this example the output directory has been specified as well as the number of blocks. In addition, the global tying option has been switched on, which means that the fragment start distributions of all isoforms within a gene share the same set of parameters.

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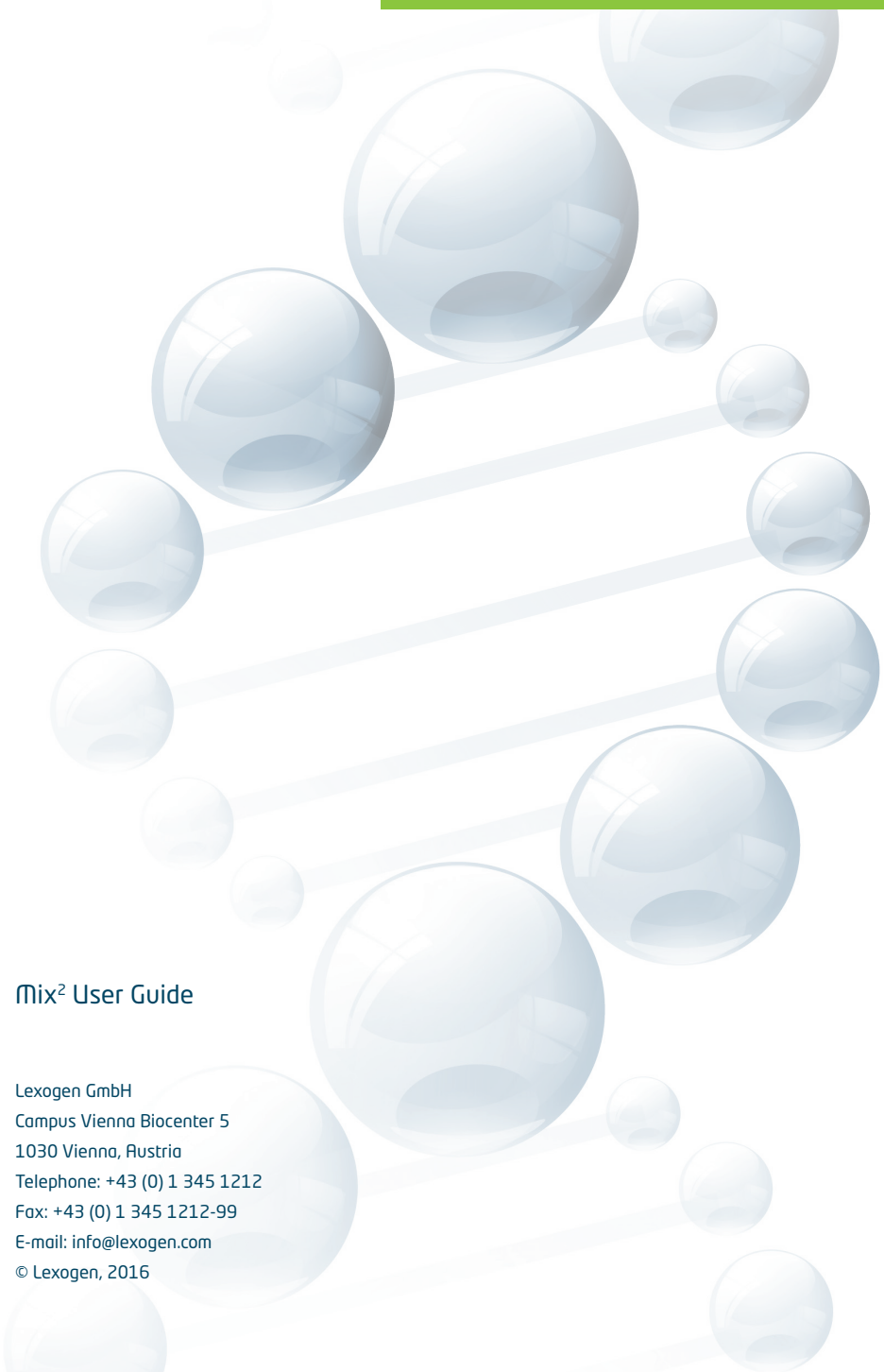

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