MetaTrans v1.0 – User Manual

Requirements

Our pipeline was developed and tested under Linux x86_64 bits. This is the list of programs and packages required to run the pipeline, therefore need to be installed:

-R software environment (R>=3.1, available here: *http://www.r-project.org/*) and these R packages with their dependencies:

R.utils
RColorBrewer
gplots
ggplot2(*)

R command to install packages: install.packages("PACKAGE_NAME")

To get the same package version used in the pipeline, here is an example for which you have to provide the url location of the wanted version:

```
packageurl <- "https://cran.r-project.org/src/contrib/Archive/R.utils/R.utils_1.34.0.tar.gz"
install.packages(packageurl, repos=NULL, type="source")
```

-Bioconducor R packages (available from *http://www.bioconductor.org/*) with dependencies:

GenomicRanges
IRanges
ShortRead
DESeq2(*)

R commands to install bioconductor packages:

```
source("https://bioconductor.org/biocLite.R") ;
biocLite("PACKAGE_NAME")
```

(*) only required for differential expression analysis

-Java Runtime Environment (JRE)

-Python: “NumPy” and "BioPython" packages. Can be easily installed from the repository with:

sudo apt-get install python-numpy
sudo apt-get install python-biopython

-USEARCH is required for the taxonomical part. A free personal copy of the 32-bit program can be downladed *here*. This pipeline was tested using USEARCH v5.2.236. To make USEARCH accessible, it can be achieved by creating a link to the binary file with the name "usearch" and making that folder accessible to the shell using these commands :

```
cd <ABSOLUTE_USEARCH_PATH> ;  chmod + x  ./<USEARCH_BINARY_FILE>
ln -s  ./<USEARCH_BINARY_FILE>  usearch
export PATH=<ABSOLUTE_USEARCH_PATH>/usearch:$PATH
```

-Programming languages that were used:
Perl(v5.14.2), Python(v2.7.3), R(R: v3.1.2; R.utils: v1.34.0; RColorBrewer: v1.1.2; gplots: v2.16.0; ggplot2: v1.0; GenomicRanges: v1.18.4; IRanges: v2.0.1; ShortRead: v1.24.0; DESeq2: v.1.6.3;), Java(v1.7.0_65-OpenJDK64), Bash(v4.2.25), Dash(v0.5.7), GNU Awk(v3.1.8), C(4.8.2), C++(v4.8.2).

Disk space requirements

Third-party software (3GB):

+ SortMeRNA(0.5MB + 3GB (indexed database files))

+ others (63.6MB)

Databases (66.1GB):

+ indexedMetaHIT14 (15.9GB)
+ indexedM5nr (36.8GB)
+ indexedGreenGenes135(10.6GB)
+ annotationsMetaHIT14 (1.6GB)
+ annotationsM5nr (240MB)
+ sortMeRNA databases (1GB)

Memory consumption

Loading MetaHIT/Greengenes indexed database into memory (at least 10GB RAM)

Quick user guide

The pipeline is configured by default to perform a functional differential expression analysis with paired-end FASTQ files without barcodes. All modules and some steps of the pipeline can be disabled individually adding these options:

-nqc (no quality control)

-nrrnarem (no rRNA removal module)

-nfunmap (no mapping of reads to functional databases)

-nsum (don't create abundance table)

-nde (don't perform a differential expression analysis with DESeq2)

However, be aware when disabling modules, bear in mind that the output of each module is passed to the following. Therefore you must be sure that if you disable a module, the input for the next module is previously processed.

For the functional analysis, the MetaHIT-2014 gene catalog database is used by default. To use the "M5nr" database this options must be supplied "-m5nr".

To perform a taxonomical analysis, two options might be used :

-tax (map read to taxonomical databases)

-sumtax (create taxonomical abundance table)

A full description of the pipeline can be found *here*.

If input is not paired-end data, additional customization of the pipeline will be required. Its implementation has been done following *POSIX* compatibility and therefore should run in any unix-like shell (list of differences between the common login shell "bash" and the Ubuntu POSIX system shell "dash" can be found *here*). Furthermore, the pipeline design is simple; it is based on a chaining of inputs and outputs between modules and tasks within each module.

Installation

The pipeline is split into three files (corresponding to pipeline scripts, third-party tools and databases) that must be downloaded and unpacked in the same folder. Please, ensure the folder name does not contain whitespaces.

tar xvf 1-Scripts.tar.gz
tar xvf 2-ThirdpartyTools.tar.gz
tar xvf 3-Databases.tar.gz

To configure proper paths to system shell in all scripts, just run this command in the pipeline's root folder where you unpacked all files (there is no need to compile):

./config

Input

All sequenced paired-end files in FASTQ format from all the samples must be placed here:

./0-Sequences

Configuration

Two files must be properly configured before running the pipeline: metadata.txt, settings.txt. Two links in the pipeline's root folder are automatically created after running "./config" to access them:

Metadata.txt

Content example:

#SampleName	SampleFiles	Shortname	Order	Type	Description	F1-Paired	F2-Diet
C1HVFACXX_8_7_1	C1HVFACXX_8_7_1.1.fastq,C1HVFACXX_8_7_1.2.fastq	C1-BF	1	mrna	H21.V3m	I1	BF
C1HVFACXX_8_7_2	C1HVFACXX_8_7_2.1.fastq,C1HVFACXX_8_7_2.2.fastq	C2-BF	2	mrna	H22.V3m	I2	BF
C1HVFACXX_8_7_3	C1HVFACXX_8_7_3.1.fastq,C1HVFACXX_8_7_3.2.fastq	P1-BF	3	mrna	P23.V3m	I3	BF
C1HVFACXX_8_7_4	C1HVFACXX_8_7_4.1.fastq,C1HVFACXX_8_7_4.2.fastq	P2-BF	4	mrna	P24.V3m	I4	BF
C1HVFACXX_8_7_5	C1HVFACXX_8_7_5.1.fastq,C1HVFACXX_8_7_5.2.fastq	C1-AF	5	mrna	H25.V3m	I1	AF
C1HVFACXX_8_7_6	C1HVFACXX_8_7_6.1.fastq,C1HVFACXX_8_7_6.2.fastq	C2-AF	6	mrna	H26.V3m	I2	AF
C1HVFACXX_8_7_7	C1HVFACXX_8_7_7.1.fastq,C1HVFACXX_8_7_7.2.fastq	P1-AF	7	mrna	P27.V3m	I3	AF
C1HVFACXX_8_7_8	C1HVFACXX_8_7_8.1.fastq,C1HVFACXX_8_7_8.2.fastq	P2-AF	8	mrna	P28.V3m	I4	AF

The following columns are required for the pipeline (whitespaces in the column names are not allowed). More columns can be added to better describe the context, but won't be used by the pipeline:

SampleName

Specify the sample names that will be used to generate intermediate results.

SampleFiles

Two filenames, separated by comma, specifying the two pair-end FASTQ files to be used for each sample. All FASTQ files must be placed into "./0-Sequences" folder.

Shortname

Sample shortnames to be used in results files.

Order

Integer number to specify the order in which samples will be processed. To **disable** samples to be processed by the pipeline use **number 0**.

Fx-<NAME> (where "x" is an integer number. E.g.: F2-Diet)

Put here all factors/conditions involved in the experiment. The factor of interest must be the last and only can have a contrast of two levels or conditions. The first sample that is going to be processed must have the control/baseline/untreated level. All these factors are processed by "DESeq2" package to perform differential expression analysis using the following R design formula: F1 + F2 + … + Fn, being Fn the factor of interest of the experiment. **"F0"** factor number is used to **disable** factors from being processed by the pipeline, i.e. any factor beginning with "F0-<NAME>" won't be included in DESeq2 design formula.

At least two samples per level within the factor of interest are required to perform a Differential Expression analysis.

Settings.txt

Contains all shell script variables required to configure the pipeline. By default user-settings are configured for a paired-end reads using 20 threads.

```
THREADS=20                  #Set max threads used.
SORTMERNA_MEMORY=5          #Set max GB of memory used by SortMeRNA (use "MAX" allows 11.7GB)
```

Run

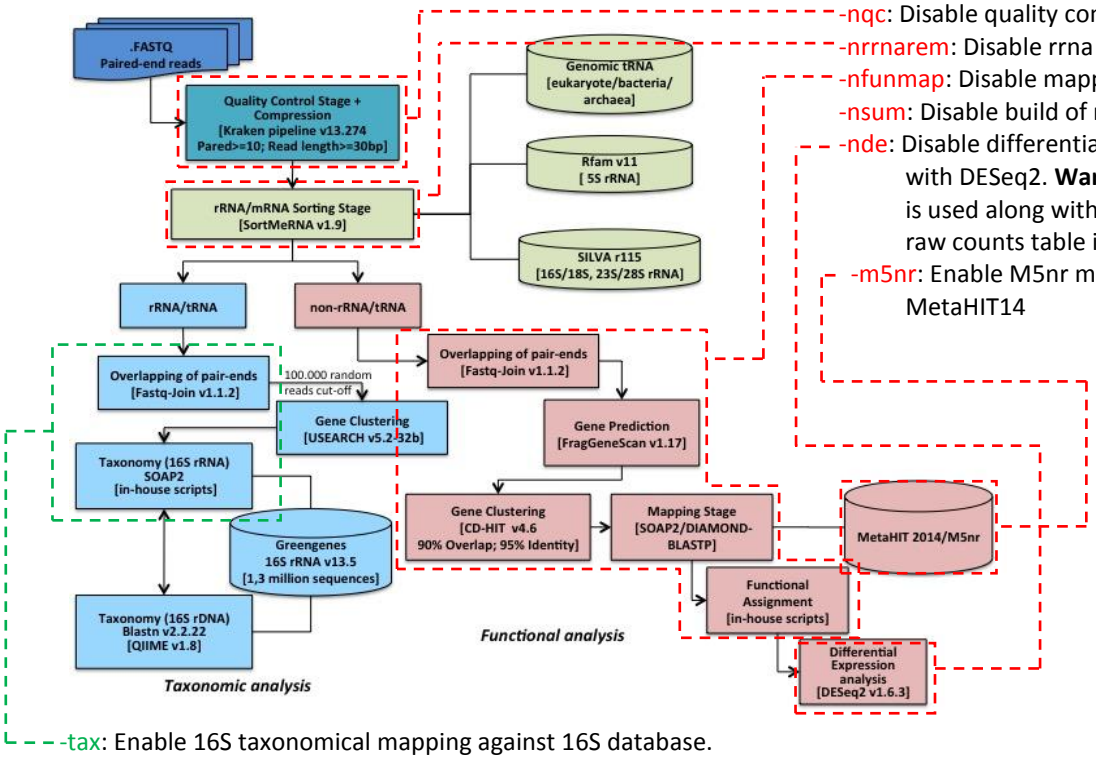
To perform a functional analysis with differential expression, one has to run:

```
./metatrans
```

Note: if you wish to re-run the QC analysis module on a previously-parsed sample, you must first remove the QC folders (m1-log,m1-temp,m1-output) ***only for that sample*** before re-running the pipeline again.

For a detailed description of all MetaTrans options run:

```
./metatrans  -h
```



-nqc: Disable quality control module.
-nrrnarem: Disable rrna removal module.
-nfunmap: Disable mapping module.
-nsum: Disable build of raw counts table.
-nde: Disable differential expression analysis with DESeq2. **Warning**: If '-nsum' option is used along with '-nde', assumes raw counts table is build and accessible.
-m5nr: Enable M5nr mapping, instead of MetaHIT14

-tax: Enable 16S taxonomical mapping against 16S database.
-sumtax: Enable build of taxonomical raw counts table.

Output

Raw counts and differential expression files produced by the functional and taxonomical analyses are placed in this directory "./5-OUTPUT":

MetaTrans-log/ : output and error messages produced by the pipeline at each run. Check "Troubleshooting" section to know more on how to find more detailed information on errors.

FunctionalAbundance/MetaHIT14-EggNOGv3/ : functional abundance summary produced by all samples of the experiment in MetaHIT14-EggNOGv3.

FunctionalAbundance/M5nr/ : functional abundance summary produced by all samples of the experiment in M5nr-EggNOG.

FunctionalAbundance/DESeq2-Analysis/funcat/ : differential expression analysis produced at COG functional category level(normalised counts, statistical test results, up/down regulated functions, PCA plot and others).

FunctionalAbundance/DESeq2-Analysis/orthids/ : differential expression analysis produced at orthologous ids level (EggNOGv3 ids) (normalised counts, statistical test results, up/down regulated functions, PCA plot and others).

TaxonomicalAbundance/Greengenes135/ : taxonomical abundance summary produced by all samples of the experiment (only available when using "-tax -sumtax" options"). A summary table is produced for each of the 7 taxonomical ranks: Kingdom(k), Phylum(p), Class(c), Order(o), Family(f), Genus(g), Species(s).

In turn, each module of the pipeline also produces these three folders (X is the module number: 1,2,3):

mX-log/: keep logs of all tasks involved in the module

mX-temp/: keep temporal files that could be used during execution

mX-output/: keep results files produced by the module. In the case of the quality control module, several reports are created within these folders:

report_fastqc_raw/ : FastQC initial report

report_fastqc_after_trimming/ : FastQC report produced after trimming stage

report_qc_reaper_after_trimming/ : reaper QC report after trimming stage produced by "Kraken tools"

report_qc_after_collapse_and_filter/ : QC report created after collapse+filter stage produced by "Kraken tools"

Uninstallation

Just remove the MetaTrans root folder where you unpacked all files.

More in detail

Additional configuration

The following sections describe more in detail some files and aspects of the pipeline that might be customized to better fit particular needs.

Settings.txt

Paired-end samples without barcodes scenario

Please note that the variables described within "settings.txt" file should be configured for each study in order to perform the quality control step using the Kraken tools, as it depends on the adaptors used when sequencing.

See *here* for a detailed description of this scenario and *here* (section "Paired end RNA sequencing") for a worked example of a pair-end scenario in Kraken tools.

- FIVEPADAPTOR, THREEPADAPTOR:

These two variables must be filled out with the adaptor sequences corresponding to the 5' adaptor and 3' adaptor, in sense orientation. Kraken will use these two sequences to trim/filter adaptors in pair-end1 reads, and will automatically complement and swap these sequences to find adaptors in the pair-end2 reads (see *here* in "5p_ad and 3p_ad" section for more detailed info).

Pair-end1 and pair-end2 reads within fastq files are assumed to be given in sense orientation (5' -> 3'). The 5' adapter will be treated as a "tabu" sequence, i.e. if present in a read the read will be discarded. There is currently no provision to simply remove the matching part. Conversely, the 3' adapter will be trimmed from the read if present.

If you do not know the 3' adapter it is possible to search for it in the input files with the program minion, which is shipped with reaper. For a FASTQ file called input.fq.gz usage is as follows:

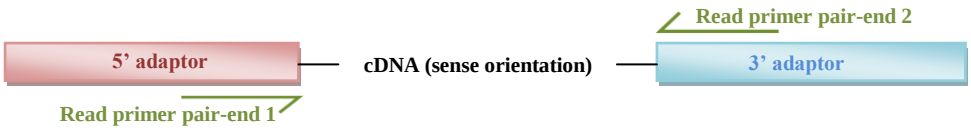
```
./0-Software/thirdpartytools/Kraken-13-274/kraken_tools_binaries/minion search-adapter  -i  input.fastq
```

By default minion will take the first 2 million reads. Use the "-do" option to change this.

E.g.:

FIVEPADAPTOR=TACACTCTTTCCCTACACGACGCTCTTCCGATCT #5' Adaptor sequence to align

THREEPADAPTOR=AGATCGGAAGAGCACACG #3' Adaptor sequence to align



5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT - cDNA (sense
READ PAIR-END1→

← READ PAIR-END2
orientation) - AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC(adaptor_barcode)ATCTCGTATGCCGTCTTCTGCTTG 3'

- TRIMSETTINGS:

A full description of all these options for the trimming/filtering process by means of the "reaper" tool is provided by Kraken tools *here*. The following descriptions are partially adapted from the original documentation.

-geom no-bc (reads geometry)

Describes the geometry of reads in fastq files. This geometry is already configured for a paired-end scenario.

-3p-global 14/2/0 (3' alignment)

Specifies best alignment found anywhere between the read and 3' adapter. Any stretch of 14 bases with at least 12 bases matches, a maximum of 2 mismatches and no gaps (indels), between 3' adapter and the read, will be considered a match. Find further information about the alignment format below.

-3p-prefix 8/2/0/0 (3' alignment)

This option specifies stringency criteria for an alignment that matches the start of the 3' adapter with the end of the read. Find further information about the alignment format below.

-3p-head-to-tail 0 (3' alignment)

This option specifies the minimum length at which a perfect match at the end of the read to the beginning of the 3' adapter should be trimmed. It is worthwhile if reads are heavily contaminated with 3' adapter sequence.

-mr-tabu 14/2/1 (5' alignment)

Match requirements for tabu sequences (sequences containing 5' adaptor). If a match is found anywhere having at least 12 bases matches, a maximum of 2 edits (mismatch/gap) and no more than one gap (indel) between 5' adapter and the read, the read is discarded. There is currently no provision to simply remove the matching part. Find further information about the alignment format below.

-clean-length 30 (length-based filtering)

After adapter matching and trimming the length of a read may be short. With -clean-length 30 any read shorter than 30

will be discarded.

-nnn-check 3/5 (N-masked bases)

Bases may not be called and show up as N in the read. This trimming test uses a sliding window to identify whether a read suffix should be removed. Any sliding window of 5 bases containing at least 3 Ns will be trimmed along with the remaining suffix of the read.

-qqq-check 43/5 (low quality check)

Low quality sequence can be detected using the median quality value in a sliding window. The quality-based trimming test is defined by the first base where this median value drops below a specified threshold. This test is applied to the full read unless an extra "/<offset>" argument is specified (see *here* for further documentation):

-qqq-check <cut-off>/ <length>/<offset>

The cutoff relates to the raw ASCII values found in the file and expressly not to the transformed P-values they represent (phred quality scores). E.g.: to set a quality Phred score cut-off of 10 in Illumina 1.8+, the value would be: 33+10 = 43

See *here* for further info on fastq phred scores.

Alignment format:

The options -3p-global, -3p-prefix, -3p-head-to-tail, -mr-tabu use an alignment format that describe the conditions that will tell if the alignment is a match or not:

l/e[/g[/o]]

mismatch=substitution=base changed by another

gap=indels (insertion or deletion)

edit=mismatch or gap

l <int> minimum length required to count sub-alignment as match. The subpart stretches

over at least l bases.

e <int> maximum allowed edit distance. There are no more than e edits in the subpart.

g <int> [optional, not active when set to 0] maximum allowed number of gaps. The total gap length in the subpart

does not exceed g.

o <int> [optional, not active when set to 0] offset:

o= 5 requires alignment to start in the first five bases of adaptor

o=-5 requires alignment to end in the last five bases of adaptor

The match occurs within an offset of o bases. The exact meaning depends on the part being matched. A zero

value implies on offset requirement at all.

- FILTERSETTINGS:

All these settings are disabled by default with "NA" (not available) value, but can be set to perform further filter or trimming. A detailed description of these options can be found *here* in the "filter" section.

FILTERSETTINGS="*low NA*

minSize NA

maxSize NA

five NA

three NA"

Not paired-end samples
In such case, the MetaTrans pipeline shell scripts should be adapted to the new scenario (the geometry of the reads would be different and should be changed for the Kraken pipeline according to *their*documentation).

FastQC contaminant list

FastQC performs detailed QC reports from raw raw sequence data coming from high throughput sequencing pipelines. For the identification of contaminant sequences it uses the following file that can be customized:

"./0-Software/config/fastqc/contaminant_list_customized.txt" which describes all contaminant sequences that will be identified in the "FastQC" reports. We can customize this file and add our specific adaptor sequences (<TAB> tabular character):

FivePrime Adaptor (custom)	<TAB>	TACACTCTTTCCCTACACGACGCTCTTCCGATCT
TrheePrime Adaptor (custom)	<TAB>	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
…		

Kraken tools quality control pipeline

For the quality control module the pipeline use an specific quality control pipeline named "SequenceImp" belonging to the "*Kraken tools*" suit. This pipeline, when used for quality control, is mainly conformed of two stages: trimming stage (namely "reaper"), which uses a program named "reaper", and a collapsing and filter stage (namely "filter"), which uses a program named "tally" to collapse reads and perform some of the filters. Three files are required to control the pipeline: a metadata file that describes the library geometry (*) of the paired-end input files, and two more files that control "reaper" (trimming/filter) and "filter" (collapsing/filter) stages.

For a further description of theses Kraken stages please check the original documentation:

-SequenceImp pipeline: *here*

-Reaper process: *here*

-Filter process: *here*

(*) a *geometry* is defined by Kraken as a description of what a read looks like, i.e. the read design.

Folder structure

The pipeline content is distributed into these folders: Databases/, Software/, Sequences/, PROCESSED_SAMPLES/ and OUTPUT/ (Fig1). Once MetaTrans finishes all sample analyses, all intermediate data that was generated by each module is saved by default into a folder for each sample. Those folders lay within PROCESSED_SAMPLES/ folder (Fig2). If "-m pipeline" option is used, a folder for each module is created instead of PROCESSED_SAMPLES/. In this case, within each module's folder a folder for each sample is created which will only contain all intermediate data created for that module (Fig3).

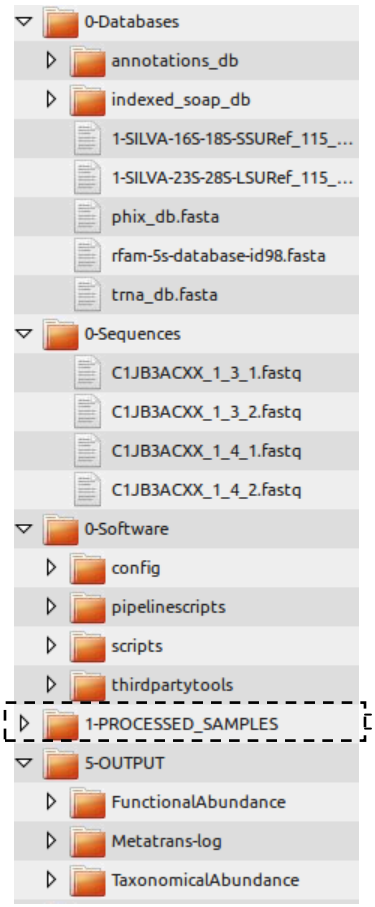


Fig1. Pipeline's folders.

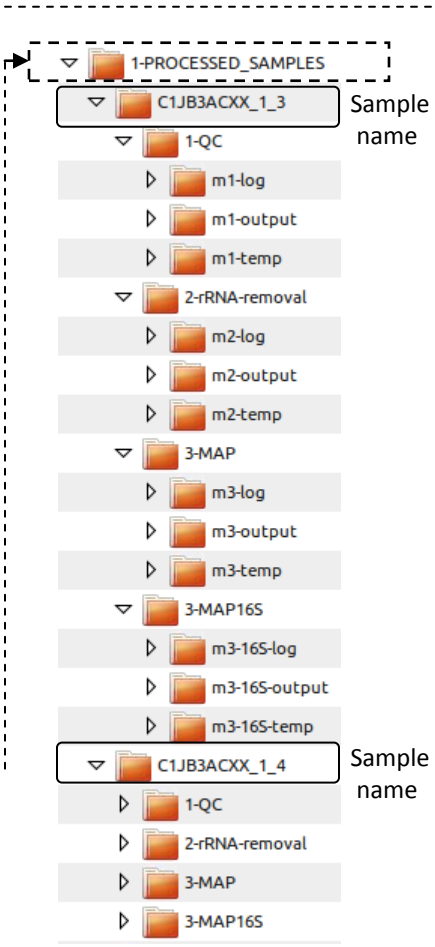


Fig2. Output structure A.
"-m sample [default mode]"
All intermediate data generated in each module hangs within each sample name.

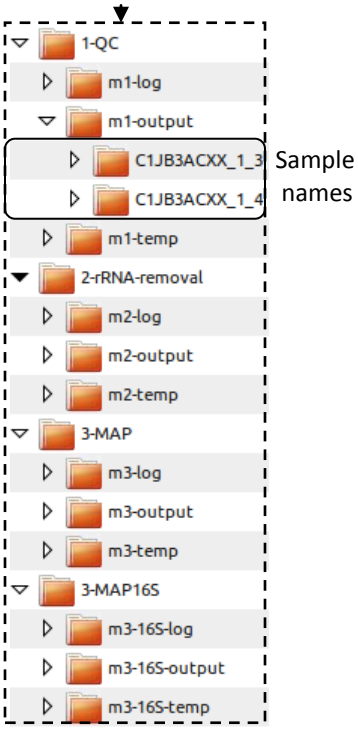


Fig3. Output structure B.
"-m pipeline" option
All intermediate data generated in each module hangs within each module name.

Troubleshooting

Finding the source of the error (help messages and log files):

In case of pipeline's errors, to trace back to the error source, one should check first the content of the last MetaTrans log in "5-OUTPUT/MetaTrans-log" (sort by "Date Modified"). To find out the meaning of some common shell error numbers (e.g. "#ERROR# Pipeline exited with error code 127") one might check this *table* or this *other* information on exit status.

If the error number is not helpful, then the next step is to identify last log files produced by the last module that was running when the error occurred, e.g.: "1-PROCESSED_SAMPLES/C1JB3ACXX_1_3/3-MAP/m3-log" (log files produced by module 3 in the sample with name "C1JB3ACXX_1_3"). Sort files by last "Date Modified" and check last logs.

If the error comes from the differential expression analysis (R script), logs are placed in: "5-OUTPUT/MetaTrans-log/*.R.log"

Re-running the pipeline after an error:

To re-run the pipeline when an error occurs, first identify from the output/logs which sample failed, then go to "1-PROCESSED_SAMPLES/<SAMPLE_NAME>/" folder and delete the entire folder of that module (e.g: "1-QC/","2-rRNA-

removal/"…). In case only failed "DE" analysis, the folder to be removed is: "5-OUTPUT/XXXAbundance/XXX/DESeq2-Analysis/".

Then modify the metadata file to run only the sample that failed (in the "Order" column set other samples to "0") and rerun "./metatrans" using the options (./metatrans -h) to disable all modules but the failing one.

In case the job performed for that particular module lasted too much time as to be entirely repeated, you may tweak the metadata file and the shellscript of the respective module to run the module just from a certain task for that particular sample (just comment out the lines of those already finished functions in the bottom of the shellscript).

Further questions or comments might be sent to:

metatrans-forum@googlegroups.com

License

This software is covered by the GPL v3 software license. For more information please see LICENSE, and COPYING files.

Third-party tools licenses and websites

- FastQC-0.10.1 (license: GPLv3 – see LICENSE.txt file ; website: *here* ; language/s: Java) (Andrews, n.d.)
- Kraken-13-274 (license: GPLv3 - see LICENSE/COPYING files; website: *here* ; language/s: Perl, Bash, C) (Davis, van Dongen, Abreu-Goodger, Bartonicek, & Enright, 2013)
- Sortmerna-1.9-linux-64-bin (license: GPLv3 – see LICENSE/COPYING files; website: *here* ; language/s: C++) (Kopylova, Noé, & Touzet, 2012)
- FastqJoinEa-utils-1.1.2-537 (license: *MIT* – see LICENSE file; website: *here* ; language/s: C) (Erik Aronesty, 2011)
- FragGeneScan-1.17 (license: GPL – see LICENSE file; website: *here* ; language/s: Perl, C) (Rho, Tang, & Ye, 2010)
- CD-HIT-4.6.1 (license: GPLv2 – see license.txt file ; website: *here* ; language/s: Perl, C++) (W Li, Jaroszewski, & Godzik, 2001) (Weizhong Li, Jaroszewski, & Godzik, 2002; Weizhong Li & Godzik, 2006; Fu, Niu, Zhu, Wu, & Li, 2012)
- SOAP-2.21 (license: GPLv3 – see COPYING file; website: *here* ; language/s: C++) (R. Li et al., 2009)
- Diamond-0.7.9 (license: specific – see COPYING file; website: *here*; language/s: C++)(Buchfink, Xie, & Huson, 2014)
- Pigz-2.3.1 (license: specific – see LICENSE/COPYING files; website: *here*; language/s: C)
- Shuf-8.22 (license: GPLv3 – see LICENSE/COPYING files ; website: *here* (within coreutils-8.22.tar.xz) ; language/s: C)

Third-party database licenses and websites

- SILVA-115 (license: *specific* – see LICENSE.txt file; website: *here*) (Quast et al., 2013)
- Greengenes-13.5 (license: *Creative Commons Attribution-ShareAlike 3.0 Unported License* – see legalcode.txt file; website: *here*) (DeSantis et al., 2006)
- Rfam-11.0 (license: *Creative Commons Zero ("CC0")* – see LICENSE file; website: *here*) (Burge, Daub, & Eberhardt, 2013)
- tRNA-all (license: unspecified; website: *here*) (Chan & Lowe, 2009)
- PhiX genome (license: public domain; NCBI website: *here*)

- MetaHIT-2014 (license: unspecified; website: *here*) (J. Li et al., 2014)
- M5nr-20130801 (license: specific – see LICENSE.md; website: *here*)(Wilke et al., 2012)

References

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Davis, M. P. A., van Dongen, S., Abreu-Goodger, C., Bartonicek, N., & Enright, A. J. (2013). Kraken: a set of tools for quality control and analysis of high-throughput sequence data. *Methods (San Diego, Calif.)*, *63*(1), 41–9. doi:10.1016/j.ymeth.2013.06.027

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Code

MetTrans is open software. If you have any comments or want to contribute to MetaTrans improvement please send an email to this *forum* :

metatrans-forum@googlegroups.com

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Citing MetaTrans

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As well as all third-party tools used by the pipeline.