SEAsnake vignette

snakemake pipeline from fastq to counts

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Contents

Introduction

SEAsnake is a snakemake pipeline to process bulk RNA-seq data from fastq sequences to gene counts. It includes the following steps. You can seen an in-depth example of how to run these steps separately outside SEAsnake in our first tutorial.

1. Quality assess sequences with FastQC
2. Remove adapters and filter low quality sequences with AdapterRemoval
   - max N = 1
   - min length = 15
   - min quality = 30
   - 5' and 3' adapters specified in config file
3. Align to reference genome with STAR
4. Quality filter alignments with samtools `view`
   - header retained
   - paired reads where both mapped
   - min MAPQ = 30

    - remove unmapped, non-primary alignments, and PCR duplicates
5. Quality assess alignments with samtools `flagstat` and/or Picard `CollectRnaSeqMetrics`
6. Count reads in genes with Subread `featureCounts`
    - exons only

Here, we provide an example of how to install and run SEAsnake on human bulk RNA-seq.

Software

Resource requirements

RAM: Indexing the human genome requires a minimum of 40 GB of RAM. If your reference genome is smaller or you already have a built index, you may be able to use less RAM. If you have many samples, you may need to use more RAM. The most common pipeline failure is running out of RAM so when in doubt, choose the larger option.

CPU: We recommend at least 1 CPU per sample to a minimum of 8 CPUs for this pipeline. More CPU usage will increase RAM usage, so if you have limited RAM, scale down the number of CPUs used.

Storage: SEAsnake itself is a small program (< 100 MB) with the vignette data adding an additional 32 GB. Thus, storage needs depend almost entirely on your sample size and sequencing depth of those samples. We recommend 65 GB of storage per 10 fastq files to be run. This comes to roughly 1 TB for 150 fastq or 75 paired-end samples.

AWS installation

SEAsnake can be pre-installed on any AWS EC2 instance.

1. Launch a new instance from the AWS console. Search 'seasnake' and select the Community AMI SEAsnake.
2. Choose your instance type keeping your resource requirements in mind. `c5` types are our most used class.
3. Leave configuration as defaults.
4. Add additional EBS storage to hold your data and results. Please leave the root storage as 16 GB; this holds the underlying SEAsnake software.
5. Tag your instance if desired.
6. Configure security as you normally would.
7. Launch the instance. This will take a couple minutes to complete before you can log-in.

Once the instance is running, log-in and complete setup as follows. First, define your AWS account information.

```
AWS_ACCESS_KEY="XXXX"
AWS_SECRET_ACCESS_KEY="XXXX"
AWS_REGION="xxxx"
```

Then, run the following script.

```
#### Basic AWS update ####
sudo yum upgrade -y
sudo yum update -y

#### Configure AWS ####
## Configure your account
export AWS_ACCESS_KEY_ID=$AWS_ACCESS_KEY
export AWS_SECRET_ACCESS_KEY=$AWS_SECRET_ACCESS_KEY
export AWS_DEFAULT_REGION=$AWS_REGION

## Setup fuse keys
```

```
echo $AWS_ACCESS_KEY:$AWS_SECRET_ACCESS_KEY > ~/.passwd-s3fs
chmod 600 ~/.passwd-s3fs

#### Setup EBS volume ####
## Get addtl volume name
## If this does not give the correct volume name, find it with lsblk
ebs_name=$(lsblk -o NAME -n -i -r | tail -n 1)
echo $ebs_name
## Format volume
sudo mkfs -t ext4 /dev/$ebs_name
## Attach SEAsnake directory to volume
sudo mount --bind /dev/$ebs_name ~/SEAsnake/
## Change permissions to read-write
sudo chmod 777 -R ~/SEAsnake/

## Clone SEAsnake from GitHub
git clone https://github.com/BIGslu/SEAsnake ~/SEAsnake
```

Other linux installation

If you are not using AWS, you can install SEAsnake and its dependencies using the following scripts.

```
#### Install conda ####
## Download conda
sudo mkdir -m 777 -p ~/apps/anaconda
cd ~/apps/anaconda
sudo curl -O https://repo.anaconda.com/archive/Anaconda3-2021.11-Linux-x86_64.sh

## Compile and install conda
sudo bash Anaconda3-2021.11-Linux-x86_64.sh -b -p /home/ec2-user/apps/anaconda -u
eval "$(/home/ec2-user/apps/anaconda/bin/conda shell.bash hook)"
conda init
sudo chmod 777 -R ~/apps/
```

Restart your terminal for conda initiation to take effect.

```
## Configure addtl conda channels
conda config --add channels bioconda
conda config --add channels conda-forge
conda config --set allow_conda_downgrades true

#### Install mamba ####
conda install -n base -c conda-forge mamba -y --repodata-fn repodata.json

#### Install SEAsnake ####
## Install git
sudo yum install git -y

## Clone SEAsnake from GitHub
sudo mkdir -m 777 ~/SEAsnake
git clone https://github.com/BIGslu/SEAsnake ~/SEAsnake

## Create environment and install SEAsnake software with mamba
mamba env create --name SEAsnake --file ~/SEAsnake/environment/Hissss_env.yaml
```

Then move your fastq data into `SEAsnake/data`.

Updating SEAsnake

If you previously installed SEAsnake, you can update it by pulling the latest version from GitHub.

```
cd ~/SEAsnake
git pull
```

SEAsnake

fastq data

Note: SEAsnake is only setup to run paired-end `fastq.qz` files in its `data/` directory.

If you are using fuse to access your fastq data on S3, link your bucket to the SEAsnake `data/` directory.

```
# Define the name of your data bucket
DATA_BUCKET="MY_BUCKET_NAME"
# Make directory for data
sudo mkdir -m 777 ~/SEAsnake/data
# Fuse bucket to directory
s3fs $DATA_BUCKET ~/SEAsnake/data \
    -o passwd_file=~/.passwd-s3fs \
    -o default_acl=public-read -o uid=1000 -o gid=1000 -o umask=0007
```

If you are transferring data by another method, create the `SEAsnake/data/` directory and copy your data there. For example, to run this vignette's data.

```
# Make directory for data
sudo mkdir -m 777 ~/SEAsnake/data
# Copy fastqs
cp ~/SEAsnake/vignette/data/*fastq.gz ~/SEAsnake/data
```

Reference genome

If you are using fuse to access your STAR-formatted genome index on S3, link your bucket to the SEAsnake `ref/` directory. *Hawn/Altman labs: These are in the bucket `human-ref`.*

```
# Define the name of your data bucket
REF_BUCKET="MY_BUCKET_NAME"
# Make directory for data
sudo mkdir -m 777 ~/SEAsnake/ref
# Fuse bucket to directory
s3fs $REF_BUCKET ~/SEAsnake/ref \
    -o passwd_file=~/.passwd-s3fs \
    -o default_acl=public-read -o uid=1000 -o gid=1000 -o umask=0007
```

If you do not have a pre-built index, the pipeline will make one for you!

Run SEAsnake

Set the number of threads you would like to use. This should be no more than your total CPU - 1. Then, activate the conda environment, which contains all pre-installed software.

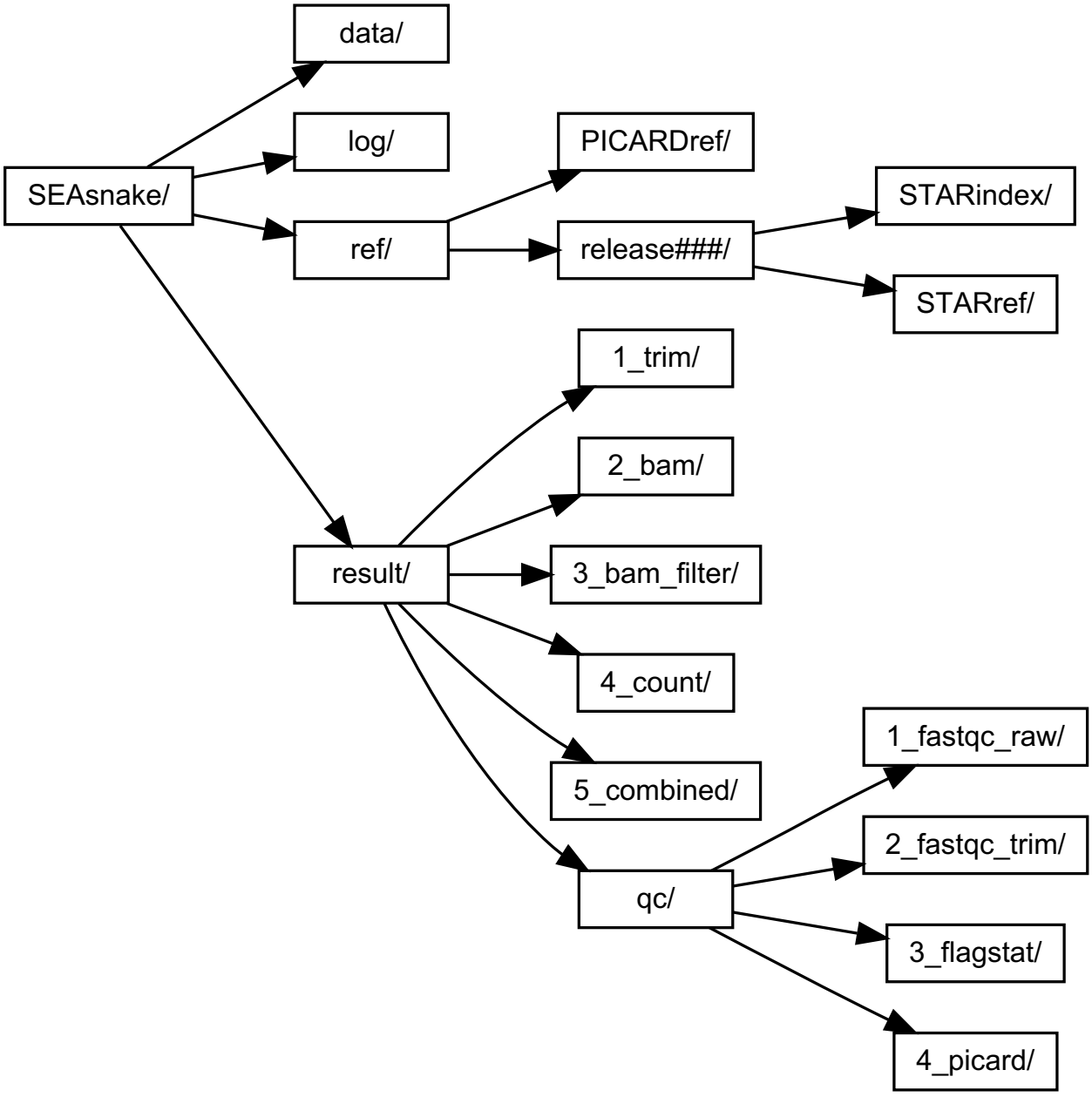
```
threads=15
conda activate SEAsnake
```

Step 1

Next, run SEAsnake step 1. This completes initial sequence quality assessment, directory structure setup, and config file creation. *Note that* ***nohup*** *and piping into a log file means nothing will appear in your terminal window. This prevents timeout and retains all messages and errors in the log.*

```
nohup snakemake --snakefile Snakefile_step1 --cores $threads >> log/SEAsnake_step1.log 2>&1
```

Your directory structure will look like



Once step 1 is complete, you will see the command prompt `$` re-appear in your console. If the terminal window times out, simply log back in and check progress in the log.

```
cat ~/SEAsnake/log/SEAsnake_step1.log
```

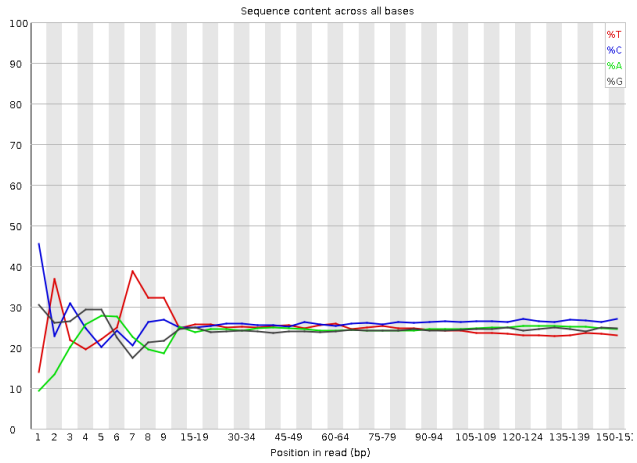
Customize config

Step 1 creates `result/config.yaml` which allows some customization of the workflow. You may wish to change some defaults. Below is an example from the vignette data with all defaults.

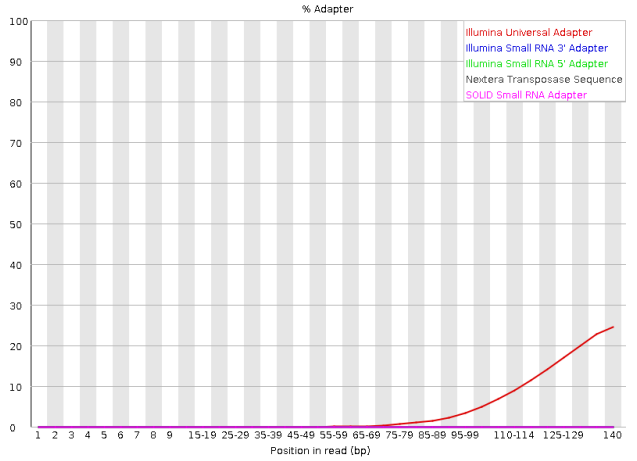
```
SampleList:
   test_S1:
    sample: 'test_S1'
    R1: 'data/test_S1_L005_R1_001.fastq.gz'
    R2: 'data/test_S1_L005_R2_001.fastq.gz'
   test_S2:
    sample: 'test_S2'
    R1: 'data/test_S2_L005_R1_001.fastq.gz'
    R2: 'data/test_S2_L005_R2_001.fastq.gz'

# Adapter removal
## Base pairs to trim from 5' end
trim5p: 10
## Removal of 3' adapter sequences? Default are Illumina Universal adapters
trimAdapt: True
adapter1: AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
adapter2: AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

# Genome alignment
## Human genome release number
release: '105'

# Alignment metrics
## Run Picard?
picard: True

# Other
threads: 15
```

Customizaton options

- SampleList
  - SEAsnake lists all `fastq.gz` from the `data/` directory and will run the pipeline for all of these samples. To remove a sample, simply remove the 4 lines relevant to that sample. To rename result files for a sample, change the "sample" field.
- Adapter removal
  - trim5p: Number of base pairs to trim from the 5' end. You determine this from FastQC "Per base sequence content". For example, this sample appears to have disproportionately high calls up to 15 bp. This supports trimming the first 15 bp of these sequences (default).



  - trimAdapt: Sequencing adapters may also exist at the 3' end. These can be seen in FastQC "Adapter content". You remove these by aligning to adapter sequences provided in the config file. Default are Illumina Universal adapters but you can provide any adapter sequence.
  - If you do not see 3' adapter contamination, **set trimAdapt to False**! You can leave the adapter sequences as is as they will not be used with the False setting. This dramatically speeds up adapter trimming.



- Genome alignment
  - Currently SEAsnake only supports human genome alignment. Specify the ENSEMBL release to use here.
- Alignment metrics
  - picard: This program provides additional alignment quality metrics listed here. If you do not need these metrics, set to False.
- threads
  - This sets the maximum number of threads to total CPU - 1 in case you forget to set it when running `snakemake`.
  - Note that Mac and Windows may not automatically fill this correctly. Please check that you have a usable thread number.

You can also find a more thorough introduction to quality assessment in our first tutorial.

Step 2

Run SEAsnake step 2. This completes adapter trimming, alignment, filtering, quality assesment, and exon counting.

```
## Run step 2 to filter, align, assess, and count sequences
nohup snakemake --snakefile Snakefile_step2 --cores $threads >> log/SEAsnake_step2.log 2>&1
```

This step will likely timeout. Because of `nohup`, you can close the terminal at any time and SEAsnake will continue to run. To check its process, simply log back in and review the log.

```
cat ~/SEAsnake/log/SEAsnake_step2.log
```

Save results

Once complete, save your `result/` and `log/` directories because these will be lost once your stop your EC2 instance. We recommend using `aws s3 sync` to save to an S3 bucket like so.

```
aws s3 sync ~/SEAsnake/result/* s3://RESULT_BUCKET
aws s3 sync ~/SEAsnake/log/* s3://RESULT_BUCKET
```

You may also wish to save your genome index for use in future runs. This saves about an hour of run time for human samples! *Hawn/Altman labs: Save to the* ***`human-ref`*** *bucket.*

```
aws s3 sync ~/SEAsnake/ref/* s3://human-ref
```

Then, close the conda environment.

```
conda deactivate
```

And un-fuse any buckets in use.

```
fusermount -u ~/SEAsnake/data
fusermount -u ~/SEAsnake/ref
```

Example results

You main results will be in `result/5_combined/` where all samples have been combined into a single table per data type.

Counts table.

```
## # A tibble: 6 x 3
##   Geneid          test_S1 test_S2
##   <chr>             <dbl>   <dbl>
## 1 ENSG00000284662       0       0
## 2 ENSG00000186827       0       0
## 3 ENSG00000186891       0       0
## 4 ENSG00000160072       0       0
## 5 ENSG00000041988       0       0
## 6 ENSG00000260179       0       0
```

Flagstat alignment metrics.

```
## # A tibble: 2 x 17
##   libID   QC_pass primary secondary supplementary duplicate primary_duplicate
##   <chr>     <dbl>   <dbl>     <dbl>         <dbl>     <dbl>             <dbl>
## 1 test_S1   11990   11990         0             0         0                 0
## 2 test_S2    5980    5980         0             0         0                 0
## # ... with 10 more variables: mapped <dbl>, primary_mapped <dbl>, paired <dbl>,
## #   read1 <dbl>, read2 <dbl>, paired_proper <dbl>, paired_mapped <dbl>,
## #   singleton <dbl>, paired_diff_chr <dbl>, paired_diff_chr5 <dbl>
```

Picard alignment metrics.

```
## # A tibble: 2 x 31
##   libID   PF_BASES PF_ALIGNED_BASES RIBOSOMAL_BASES CODING_BASES UTR_BASES
##   <chr>      <dbl>            <dbl> <lgl>                 <dbl>     <dbl>
## 1 test_S1  1656408          1638795 NA                   797841    490355
## 2 test_S2   825848           816834 NA                   392505    246977
## # ... with 25 more variables: INTRONIC_BASES <dbl>, INTERGENIC_BASES <dbl>,
## #   IGNORED_READS <dbl>, CORRECT_STRAND_READS <dbl>,
## #   INCORRECT_STRAND_READS <dbl>, NUM_R1_TRANSCRIPT_STRAND_READS <dbl>,
## #   NUM_R2_TRANSCRIPT_STRAND_READS <dbl>, NUM_UNEXPLAINED_READS <dbl>,
## #   PCT_R1_TRANSCRIPT_STRAND_READS <dbl>, PCT_R2_TRANSCRIPT_STRAND_READS <dbl>,
## #   PCT_RIBOSOMAL_BASES <lgl>, PCT_CODING_BASES <dbl>, PCT_UTR_BASES <dbl>,
## #   PCT_INTRONIC_BASES <dbl>, PCT_INTERGENIC_BASES <dbl>, ...
```

Contribute to SEAsnake

SEAsnake is an open source workflow. We would love your feedback including error/bug reports and requests for additional features! Please let us known on our GitHub. We also welcome community code additions through pull requests!

Cite SEAsnake

Coming soon!
