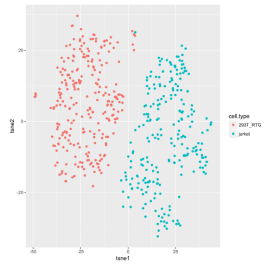
Jun 27, 2019

# Instructional tutorial for using ***demuxlet***



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Hyun Min Kang[1], Meena Subramaniam[2], Sasha Targ[2], Michelle Nguyen[2], Lenka Maliskova[2], Elizabeth McCarthy[2], Eunice Wan[2], Simon Wong[2], Lauren Byrnes[2], Cristina M Lanata[2], Rachel E Gate[2], Sara Mostafavi[3], Alexander Marson[2], Noah Zaitlen[2], Lindsey A Criswell[2], Chun Jimmie Ye[2]

[1]Department of Biostatistics and Center for Statistical Genetics, University of Michigan; [2]University of California, San Francisco; [3]Department of Statistics, University of British Columbia

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**External link: https://github.com/statgen/demuxlet/tree/master/tutorial**

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**We use this protocol and it's working**

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## Abstract

**Genetic multiplexing of barcoded single cell RNA-seq**

**Introduction**

***demuxlet*** is a software tool to deconvolute sample identity and identify multiplets when multiple samples are pooled by barcoded single cell sequencing.***demuxlet*** takes (1) a SAM/BAM/CRAM file produced by the standard 10x sequencing platform, or any other barcoded single cell RNA-seq (with proper --tag-UMI and --tag-group) options (2) a VCF/BCF file containing the genotype (GT), posterior probability (GP), or genotype likelihood (GL) to assign each barcode to a specific sample (or a pair of samples) in the VCF file.

**Tutorial for demuxlet**

This tutorial provides streamlined instructions for using the tool **demuxlet**. For a more detailed description of all of the options available to use with **demuxlet**, please refer to the README (attached in .zip file). **demuxlet** has several dependencies and this tutorial provides steps to use a docker instance to minimize compatibility issues and an alternative of installing demuxlet from source.

**demuxlet** is a software tool to deconvolute sample identity and identify multiplets when multiple samples are pooled by barcoded single cell sequencing. **demuxlet** requires the following input files:

1. a SAM/BAM/CRAM file produced by the standard 10x sequencing platform, or any other barcoded single cell RNA-seq (with proper **--tag-UMI** and **--tag-group**) options.
2. a VCF/BCF file containing the genotype (GT), posterior probability (GP), or genotype likelihood (GL) to assign each barcode to a specific sample (or a pair of samples) in the VCF file.

## Attachments




tutorial.zip
2.7MB

## Guidelines

**Additional resources**

The README for demuxlet is available **here**.
If you have questions about using demuxlet or suggestions for future releases, please contact **jimmie.ye@ucsf.edu**.

**Source of 293T and Jurkat VCF file.**

1. 293T VCF Source website: http://hek293genome.org/v2/data.php Source file:http://bioinformatics.psb.ugent.be/downloads/genomeview/hek293/SNP/293T_RTG.vcf.gz
2. Jurkat VCF Source website: https://zenodo.org/record/400615#.WYIh7IQrLIV Source file: https://zenodo.org/record/400615/files/jurkat_final_variant_calls.tar.gz
3. 293T:jurkat VCF file generation We used the CrossMap tool to liftover the 293T vcf file from hg18 to hg19. The tetraploid genotype for the Jurkat vcf was collapsed to a diploid genotype before being merged with the 293T vcf file and the resulting file was filtered to contain only the exon positions.

**Installing demuxlet**

**1. MAC NOTES**

You will need to install some additional packages on a Mac.

First, you will need XCode CommandLineTools

Second, it's good to install homebrew:

```
/usr/bin/ruby -e "$(curl -fsSL
https://raw.githubusercontent.com/Homebrew/install/master/install)"
```

Then, we need to install autoconf, automake, and libtool

```
$ brew install autoconf automake libtool
```

We will also need a new clang since Apple's clang doesn't support OpenMPI

```
$ brew install llvm
$ export CC=/usr/local/Cellar/llvm/4.0.0_1/bin/clang
$ export CXX=/usr/local/Cellar/llvm/4.0.0_1/bin/clang++
$ export LDFLAGS=-L/usr/local/Cellar/llvm/4.0.0_1/lib/
```

The CRAM format may use LZMA2 compression, which is implemented in HTSlib by using compression routines from liblzma http://tukaani.org/xz/.

Building HTSlib requires liblzma development files to be installed on the build machine; you may need to ensure a package such as liblzma-dev (on Debian or Ubuntu Linux), xz-devel (on RPM-based Linux distributions or Cygwin), or xz (via Homebrew on macOS) is installed; or build XZ Utils from source. Assuming we are on a Mac:

```
$ brew install xz
```

**2. Install htslib**

```
$ git clone https://github.com/samtools/htslib.git
$ cd path/to/htslib
$ autoheader
$ autoconf
$ ./configure # optional, --prefix=/path/file
$ make
$ make install # optional, DESTDIR=/path/file
```

**3. Install demuxlet**

demuxlet and htslib should be installed in same directory

```
$ git clone https://github.com/statgen/demuxlet.git
$ cd /path/to/demuxlet
$ mkdir m4
$ autoreconf -vfi
$ ./configure # optional, --prefix=/path/file
$ make
$ make install # optional, DESTDIR=/path/file
```

**Tips for running**

- Set **--alpha 0 --alpha 0.5**, which assumes the expected proportion of 50% genetic mixture from two individuals, to get better estimates of doublets.
- Set **--group-list** to a list of barcodes (i.e. barcodes.tsv from 10X) to speed things up and only get demultiplexing for cells called by other methods
- To reproduce the results presented in Figure 2 of the demuxlet paper, please go to: https://github.com/yelabucsf/demuxlet_paper_code/tree/master/fig2 to download the vcf and the outputs of demuxlet.

**Using demuxlet**

demuxlet uses a self-documentation utility. You can run each utility with -man or -help option to see the command line usages.

```
$ ./demuxlet          (for short usage)
$ ./demuxlet -help    (for detailed usage)
```

The detailed usage is also pasted below.

```
Options for input SAM/BAM/CRAM
   --sam           [STR: ]            : Input SAM/BAM/CRAM file. Must be sorted by
coordinates and indexed
   --tag-group     [STR: CB]          : Tag representing readgroup or cell barcodes,
in the case to partition the BAM file into multiple groups. For 10x genomics, use CB
   --tag-UMI       [STR: UB]          : Tag representing UMIs. For 10x genomiucs, use
UB

Options for input VCF/BCF
   --vcf           [STR: ]            : Input VCF/BCF file, containing the individual
genotypes (GT), posterior probability (GP), or genotype likelihood (PL)
   --field         [STR: GP]          : FORMAT field to extract the genotype,
likelihood, or posterior from
   --geno-error    [FLT: 0.01]        : Genotype error rate (must be used with --
field GT)
   --min-mac       [INT: 1]           : Minimum minor allele frequency   --min-
callrate  [FLT: 0.50]         : Minimum call rate
   --sm            [V_STR: ]          : List of sample IDs to compare to (default:
use all)
   --sm-list       [STR: ]            : File containing the list of sample IDs to
compare

Output Options
  --out           [STR: ]            : Output file prefix
  --alpha         [V_FLT: ]          : Grid of alpha to search for (default is 0.1,
0.2, 0.3, 0.4, 0.5)
  --write-pair    [FLG: OFF]         : Writing the (HUGE) pair file
  --doublet-prior [FLT: 0.50]        : Prior of doublet
  --sam-verbose   [INT: 1000000]     : Verbose message frequency for SAM/BAM/CRAM
  --vcf-verbose   [INT: 10000]       : Verbose message frequency for VCF/BCF

Read filtering Options
   --cap-BQ        [INT: 40]          : Maximum base quality (higher BQ will be
capped)
   --min-BQ        [INT: 13]          : Minimum base quality to consider (lower BQ
will be skipped)
   --min-MQ        [INT: 20]          : Minimum mapping quality to consider (lower MQ
will be ignored)
   --min-TD        [INT: 0]           : Minimum distance to the tail (lower will be
ignored)
   --excl-flag     [INT: 3844]        : SAM/BAM FLAGs to be excluded

Cell/droplet filtering options
```

```
   --group-list    [STR: ]             : List of tag readgroup/cell barcode to
consider in this run. All other barcodes will be ignored. This is useful for
parallelized run
   --min-total     [INT: 0]            : Minimum number of total reads for a
droplet/cell to be considered
   --min-uniq      [INT: 0]            : Minimum number of unique reads (determined by
UMI/SNP pair) for a droplet/cell to be considered
   --min-snp       [INT: 0]            : Minimum number of SNPs with coverage for a
droplet/cell to be considered
```

**Interpretation of output files**

***demuxlet*** generates multiple output file, such as **[prefix].best**, **[prefix].sing**, **[prefix].sing2**, and optionally **[prefix].pair** (with **--write-pair** argument). Each file contains the following information

- The **[prefix].best** file contains the best guess of the sample identity, with detailed statistics to reach to the best guess
- The **[prefix].sing** file contains the statistics for matching each cell with each possible sample.
- The **[prefix].sing2** file contains the statistics similar information to the previous one, but generated for sanity checking of the **[prefix].pair** results.
- The **[prefix].pair** file contains the statistics for matching each cell with each possible configuration of doublet.

The **[prefix].best** file contains the following 22 columns.

1. BARCODE - Cell barcode for the cell that is being assigned in this row
2. RD.TOTL - The total number of reads overlapping with variant sites for each droplet.
3. RD.PASS - The total number of reads that passed the quality threshold, such as mapping quality, base quality.
4. RD.UNIQ - The total number of UMIs that passed the quality threshold. If a UMI is observed in a single variant multiple times, it won't be counted more. If a UMI is observed across multiple variants, it will be counted as different.
5. N.SNP - The total number of variants overlapping with any read in the droplet.
6. BEST - The best assignment for sample ID.
   - For singlets, SNG-
   - For doublets, DBL---
   - For ambiguous droplets, , AMB---<doublet ID1/ID2>)
7. SNG.1ST - The best singlet assignment for sample ID
8. SNG.LLK1 - The log(likelihood that the ID from SNG.1ST is the correct assignment)
9. SNG.2ND - The next best singlet assignment for sample ID
10. SNG.LLK2 - The log(likelihood that the ID from SNG.2ND is the correct assignment)
11. SNG.LLK0 - The log-likelihood from allele frequencies only
12. DBL.1ST - The sample ID that is most likely included if the assignment is a doublet

13. DBL.2ND - The sample ID that is next most likely included ifthe assignment is a doublet
14. ALPHA - % Mixture Proportion
15. LLK12 - The log(likelihood that the ID is a doublet)
16. LLK1 - The log(likelihood that the ID from DBL.1ST is the correct singlet assignment)
17. LLK2 - The log(likelihood that the ID from DBL.2ND is the correct singlet assignment)
18. LLK10 - The log(likelihood that the ID from DBL.1ST is one of the doublet, and the other doublet identity is calculated from allele frequencies only)
19. LLK20 - The log(likelihood that the ID from DBL.2ND is one of the doublet, and the other doublet identity is calculated from allele frequencies only)
20. LLK00 - The log(likelihood that the droplet is doublet, but both identities are calculated from allele frequencies only)
21. PRB.DBL - Posterior probability of the doublet assignment
22. PRB.SNG1 - Posterior probability of the singlet assignment when excluding all possible doublets

## Troubleshooting

## Before start

Add bam file name for **$bam** and vcf file name for **$vcf**. Use **<(zcat $vcf)** or **<(gzcat $vcf)** if vcf file is compressed.

The options for **--field** are individual genotypes (GT), posterior probability (GP), or genotype likelihood (PL). If using GT **--field** option for, you must include **--geno-error**, which is the genotype error rate.

#### demuxlet output

The demuxlet software produces 3 output files. Among those, most important are:

1. **[prefix].best** The .best file contains the assignments of the best sample identity (singlet: **SNG-**; doublet: **DBL-**; ambiguous: **AMB-< >**) in the **BEST** column for each cell barcode identified in the **BARCODE** column along with details of the statistics used to determine the best identity.
2. The **[prefix].single** file contains the statistics for matching each cell with each possible sample

For complete descriptions of the generated files and columns in the output, please see the **Guidelines**.

1 Please select between the two following options:

1. Installating from Docker
2. Installing from source

STEP CASE

## Installating from Docker 6 steps

Select this option for docker installation.

2 **Installing docker**

First, get docker for whatever platform you feel comfortable with: https://www.docker.com/get-started

3 **Running demuxlet from docker**

We have created a docker container at **yimmieg/demuxlet** to run demuxlet through docker.

You can run it with

```
$ docker run -v path/to/tutorial:/data yimmieg/demuxlet --sam
/data/$bam --vcf /data/$vcf --field $(GT or GP or PL) --out
/data/$filename
```

## Analyzing the sample dataset

4 **Create directory and download datasets**

Now, let's first download the data we need. We are now providing a one-stop-shop to download all of the data you need for the tutorial: https://ucsf.box.com/s/vg1bycvsjgyg63gkqsputprq5rxzjl6k. After downloading and unzipping (it's BIG), you should have a directory called **demuxlet.tutorial**.

5 **Run demuxlet**

```
$ cd demuxlet.tutorial
$ docker run -v ./:/data yimmieg/demuxlet --sam
/data/jurkat_293t_downsampled_n500_full_bam.bam --vcf
/data/jurkat_293t_exons_only.vcf --field GT --out
data/jurkat_293t_demuxlet
```

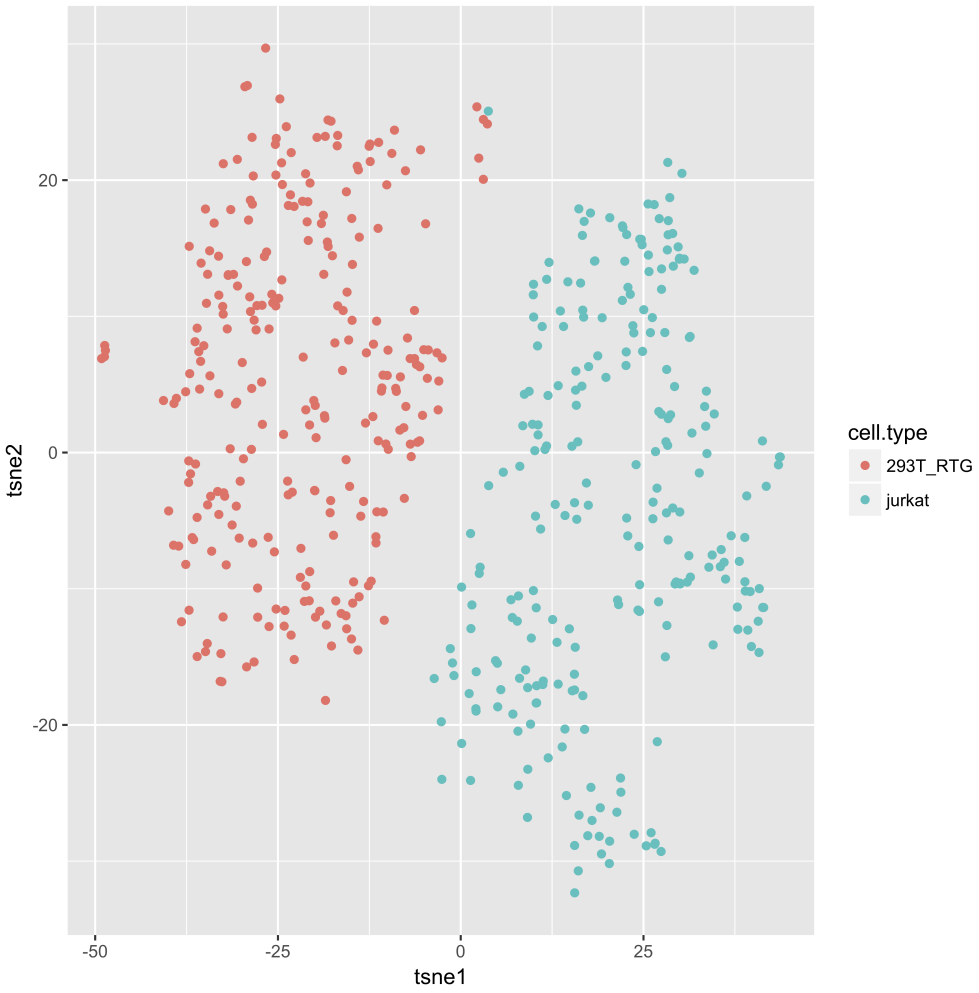
6 **Compare the called genotypes vs transcriptome data**

In this analysis, we will use R to produce a t-SNE (t-Distributed Stochastic Neighbor Embedding) plot of the cells from the 293T:Jurkat 10x experiment with the cells colored by the assignments from the demuxlet pipeline.

```
library(ggplot2);
library(data.table);

## let's read in the barcodes
tsne <- fread("analysis_csv/tsne/projection.csv");
demuxlet <- fread("jurkat_293t_demuxlet.best");

## let's filter for the barcodes that we sampled

df <- data.frame(tsne1=tsne$"TSNE-1"
[na.omit(match(demuxlet$BARCODE,tsne$Barcode))], tsne2=tsne$"TSNE-
2"[na.omit(match(demuxlet$BARCODE,tsne$Barcode))],
doublet=sapply(demuxlet$BEST,function(x){strsplit(x,"-")[[1]]
[[1]]}), cell.type=sapply(demuxlet$BEST,function(x){strsplit(x,"-
")[[1]][[2]]}))

ggplot(aes(tsne1,tsne2,color=cell.type),data=df)+geom_point()
```

Expected result

After this, you should get the following image:




7 Let's also take a look at the doublets.

```
ggplot(aes(tsne1,tsne2,color=doublet),data=df)+geom_point()
```

## Expected result

After that, you should get the following image: