



# TRANSECT Manual 24.04

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# 1. Background

---

TRANSECT works by defining two groups (strata, plural for stratum) within a cohort based solely on the expression of a gene or a gene set of interest and subsequently compares the stratum, one against the other, for global expression changes and functional differences. TRANSECT outputs descriptive statistics about the gene/s of interest, the products of the stratification process, the differential expression results and subsequent enrichment outcomes. The application uses publicly available large cohort datasets and simply requires the user to choose at a minimum

1. the **cohort database** containing participant IDs and gene expression measurements
2. a **gene (or multiple genes)** of interest whose expression levels are used to rank participants in the cohort database
3. an **integer percentile** value used on the expression or ranking measurements as a threshold to partition the cohort into low and high stratum for subsequent comparisons

# 2. Getting started

---

## 2.1 Installation using Conda

(10 - 15 minutes on an average PC)

Making use of a Conda environment for the sizable number of prerequisite modules and dependencies needed by TRANSECT is recommended for most use cases. It is not only easier to achieve but cleaner, simpler to manage and way quicker than the native install

1. Start by cloning the repository using the git command to a suitable location on your device

```
$ git clone https://github.com/twobeachers75/TRANSECT.git
```

Alternatively TRANSECT code and executable files can be downloaded from GitHub at <https://github.com/twobeachers75/TRANSECT>. Click on the green "Code" button followed by "Download ZIP" (note the download location). Find the downloaded ZIP file and move it to an appropriate location if required, before extracting the contents and renaming the folder

```
$ unzip TRANSECT-main.zip  
$ mv TRANSECT-main TRANSECT
```

2. Install Conda on your system (version > 24.1.0). You can skip this step if you already have it. There are many wikis on how to install Conda for Ubuntu, [here](#) is just one. Please consult the Conda documentation relevant to your operating system.

```

### follow the instructions outlined in the link above which should look
something like this for Linux
$ mkdir -p ~/miniconda3
$ wget https://repo.anaconda.com/miniconda/Miniconda3-latest-Linux-
x86_64.sh -O ~/miniconda3/miniconda.sh
$ bash ~/miniconda3/miniconda.sh -b -u -p ~/miniconda3
$ rm -rf ~/miniconda3/miniconda.sh

### don't forget to initialize the bash shell. Mac users need to check
their default shell and change the following command appropriately
$ ~/miniconda3/bin/conda init bash

### Afterwards, you will be asked to restart your terminal whereby you
should see (base) at the prompt. Ignore it for now.

```

3. Next, we create the TRANSECT Conda environment. Here we will run an installation script that automates the creation of the TRANSECT environment, with all the tools and dependencies required to run TRANSECT. The scripts required for this can be found in the INSTALL/ subdirectory of the TRANSECT folder.

```

### change into the top directory of the downloaded folder (TRANSECT) and
navigate to the INSTALL folder
$ cd <path to>/TRANSECT/INSTALL

### First, run the conda install script
$ ./TRANSECT_conda_install.sh

### Next, upon successful completion of the previous step, activate the
newly created environment
$ conda activate TRANSECT

### Finally, whilst still within the TRANSECT/INSTALL directory and in the
TRANSECT environment run the post installation script to complete the setup
$ ./TRANSECT_post_conda_install.sh

### You now need to reactivate the TRANSECT environment to apply the
changes.
$ conda deactivate
$ conda activate TRANSECT

```

**And that's it!** You should now have all the necessary applications and dependencies in the TRANSECT environment to run this application. Please note, just like any virtual environment you are required to activate the TRANSECT environment in order to use the application. You can deactivate at will when not in use.

A few extra very useful commands for those not accustomed to Conda environments

```

### By default, Conda auto-activates the "base" default environment so each
time you open a terminal you will automatically be in the (base) environment. I
prefer not to have this happen. To disable, run the following commands.

```

```
#First, deactivate any environment until there is no (XXX) at the beginning of
the prompt, then turn off auto_activate_base
$ conda deactivate
$ conda config --set auto_activate_base false

### To check what environments you have on your system
$ conda env list
### You should see "base" and "TRANSECT" at the very least

### To activate TRANSECT at any time
$ conda activate TRANSECT
### Once you have finished, to deactivate the TRANSECT environment
$ conda deactivate

### To remove/uninstall the TRANSECT environment
conda remove -n TRANSECT -all
```

More information about managing Conda environment can be found [here](#)

## 2.2 Native installation on Ubuntu

(30 - 45 minutes or longer on an average PC)

**NOTE: This is not the recommended installation procedure.** TRANSECT requires and depends on numerous packages and applications. These take some time to install natively if not already present. A fresh install on a vanilla Ubuntu 22.04 can take 30-45mins depending on the PC and network speeds.

1. To start, clone the repo

```
$ git clone https://github.com/twobees75/TRANSECT.git
```

Alternatively TRANSECT code and executable files can be downloaded from GitHub at <https://github.com/twobees75/TRANSECT>. Click on the green "Code" button followed by "Download ZIP" (note the download location). Find the downloaded ZIP file and move it to an appropriate location if required, before extracting the contents and renaming the folder

```
$ unzip TRANSECT-main.zip
$ mv TRANSECT-main TRANSECT
```

2. Install python3 pip, java if required, and other TRANSECT dependencies  
(approx. 1-2min)

```

### change into the top directory of the downloaded folder (TRANSECT)
$ cd <path to>/TRANSECT

### install pip and other deb requirements
$ sudo apt install python3-pip default-jre libfontconfig1-dev libcurl4-
openssl-dev libssl-dev libxml2-dev libharfbuzz-dev libfribidi-dev
libfreetype6-dev libpng-dev libtiff5-dev libjpeg-dev pandoc

### install python modules
$ python3 -m pip install -r pip_requirements.txt

```

3. Install R, the "pacman" package and Bioconductor specific packages. You can skip this step if you already have R. There are many wikis on how to install R on Ubuntu, [here](#) is just one (specifically for Ubuntu 22.04) (*approx. 1min*)

```

# follow the instructions outlined in the link above which should look
something like this
$ wget -qO- https://cloud.r-
project.org/bin/linux/ubuntu/marutter_pubkey.asc | sudo gpg --dearmor -o
/usr/share/keyrings/r-project.gpg
$ echo "deb [signed-by=/usr/share/keyrings/r-project.gpg] https://cloud.r-
project.org/bin/linux/ubuntu jammy-cran40/" | sudo $ tee -a
/etc/apt/sources.list.d/r-project.list
$ sudo apt update
$ sudo apt install r-base

```

4. Start R from the terminal and install pacman and devtools. Follow the prompts and choose (if asked) to install these packages into a personal library.

Once you enter the R shell you should see printed out in the terminal a number of lines about the R version and licenses followed by a ">" symbol. I have used this symbol below to indicate that you need to be in the R shell to run these commands but, you can't copy the ">" symbol too. It won't work. \*(approx. 25mins)

```

### start R
R
> install.packages(c("pacman", "devtools"))
# Note: maybe wise here to go get a coffee as the previous command takes
quite some time to finish! (approx. 15mins)

### whilst still in the R environment, load devtools and install rlogging
> library("devtools")
> install_github("https://github.com/mjkallen/rlogging.git")

### also install required Bioconductor packages
> if (!require("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
> BiocManager::install(version = "3.19")
> BiocManager::install(c("edgeR", "Glimma", "DEFormats"))
# Note: probably time for another coffee. Sorry! (approx. 10mins)

```

```

#### once successfully completed you can quit R, no need to save the
workspace.
# No more coffee for you today ;-
> q()

```

NOTE: TRANSECT requires many additional R packages however these are all installed on demand the first time (and only the first time) you run each one of the different TRANSECT commands after a nativ install. Please keep this in mind on your first run as it will take substantially longer compared to all subsequent runs.

## 3. Basic demonstration

---

TRANSECT has two main operations; **Prepare** and **Analyse**.

In order for TRANSECT to function, it first requires a cohort dataset to work on. This is retrieved and formatted appropriately by the **Prepare** scripts. Subsequently, TRANSECT can run analyses on the downloaded data using the **Analyse** scripts

Example commands to investigate ZEB1 using the RECOUNT3 TCGA PRAD cohort;

```

### First, if not already make sure to activate the TRANSECT environment
conda activate TRANSECT

### Next Download the RECOUNT3 PRAD data (approx. 3mins)
# run the RECOUNT3 prepare script for TCGA-PRAD (the resulting files can be
found in <path to>/TRANSECT/data/RECOUNT3/PRAD)
R3_prepare_directories.sh -p PRAD
# NOTE: you only need to do this one time per cohort dataset. Once you have it
you don't need to download it again!

### Finally, Run the TRANSECT analysis
# TRANSECT saves output in the current working folder so best to create a new
folder specifically for each run
cd <path to>/TRANSECT/output/RECOUNT3
mkdir -p ZEB1_PRAD_test
cd ZEB1_PRAD_test

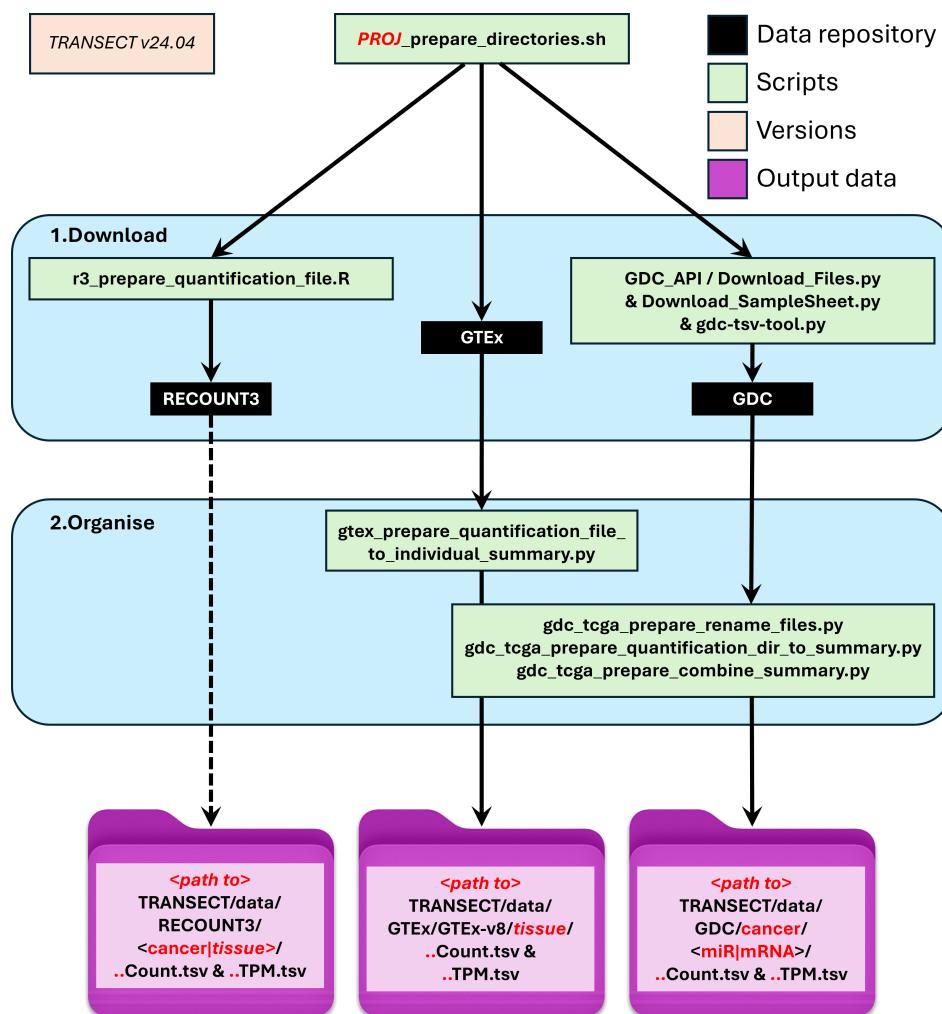
# Now, run the RECOUNT3 analyse script using the PRAD data we just retreived,
investigating the gene ZEB1, partitioning the participants using a percentile
threshold of 5, with all outputs.
R3_analyse_GOI.sh -p PRAD -g ZEB1 -s mRNA -t 5 -a

### Done! Explore the outputs. Try swapping ZEB1 for your favourite gene
(remember, new folder for each run).

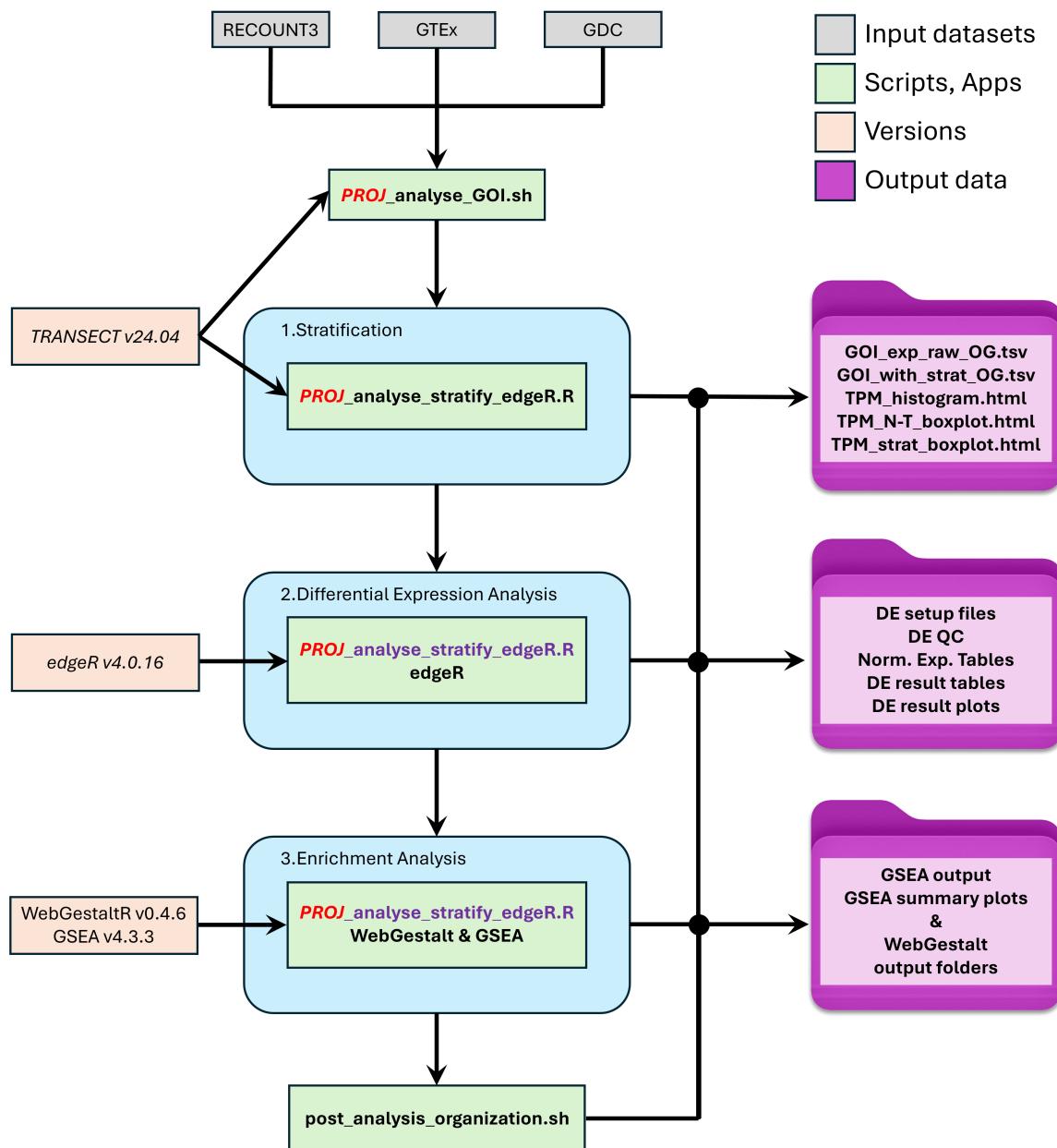
```

## 4. TRANSECT workflow

### 4.1 Prepare workflow diagram



## 4.2 Analyse workflow diagram



## 5. Stratification modes

The basic premise of TRANSECT is to stratify individuals from large cohort transcriptomic data into defined groups called strata based on singular gene expression or composite gene expression sets. The stratified participant strata are subsequently compared one to the other in order to assess global expression changes and functional differences.

### 5.1 Single gene analysis

Single gene stratification is simply the division of individuals within a cohort population into distinct strata based solely on the expression of one single gene. In the current version of TRANSECT, individuals with expression levels at or near both ends of the physiological limits for the gene of interest are grouped separately and subsequently compared (depiction below).

### Expression data table (participants x gene)

	GOI	gene-b	gene-c	gene-d	gene-e	gene-f	gene...	gene-z
participant15	1.0	68.2	20.8	94.5	56.5	36.9	.....	47.6
	2.0	14.8	93.3	40.3	23.6	46.7	.....	95.3
	3.0	88.0	69.4	87.4	74.8	47.5	.....	46.5
	4.0	65.1	96.4	39.9	65.2	70.7	.....	83.5
	5.0	14.1	95.7	7.4	41.8	4.2	.....	29.4
participant8	6.0	51.2	91.2	53.1	89.8	16.8	.....	7.7
participant10	7.0	55.6	71.2	17.4	46.4	39.2	.....	86.2
participantx	8.0	87.5	1.1	59.7	56.9	26.8	.....	31.6
participant4	9.0	7.0	40.2	33.0	48.7	17.1	.....	19.6
participant16	10.0	75.2	51.2	61.9	94.8	39.8	.....	33.2
participant14	11.0	63.5	26.1	8.3	26.1	51.6	.....	49.3
participant1	12.0	6.7	44.3	24.9	30.3	19.6	.....	75.5
participant18	13.0	59.5	22.5	99.9	58.8	98.6	.....	43.7
participant7	14.0	16.9	15.5	49.9	81.2	44.9	.....	82.6
participant5	15.0	16.6	48.5	98.9	75.7	62.5	.....	39.0
participant12	16.0	16.5	48.3	86.6	17.8	15.9	.....	24.7
participant9	17.0	23.7	14.1	93.6	32.9	2.8	.....	49.7
participant13	18.0	14.5	57.7	29.9	95.8	38.2	.....	41.5
participant....	.....	.....	.....	.....	.....	.....	.....	.....
participant-n	20.0	41.6	87.5	83.5	39.0	60.3	.....	50.1

LOW

HIGH

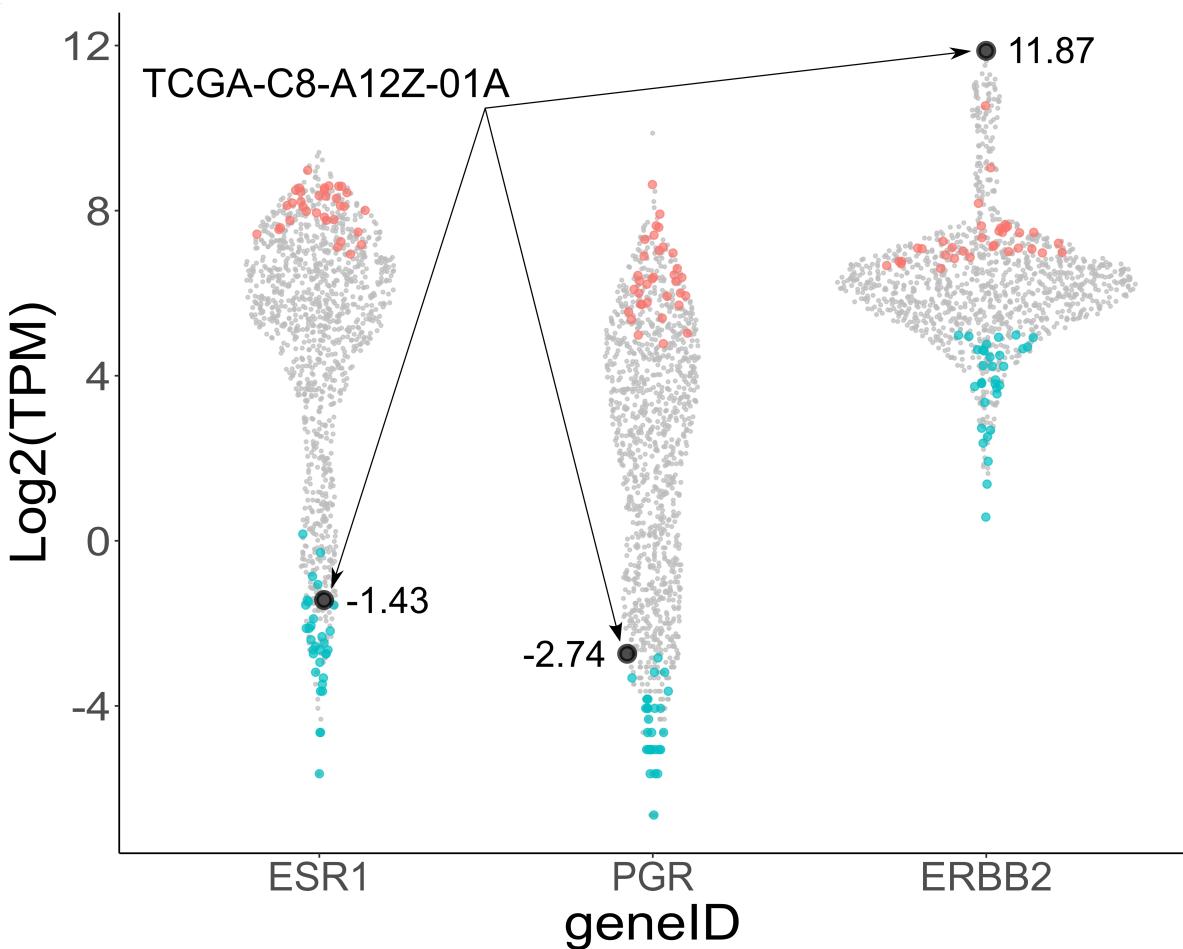
1. Each row contains expression measurements from a single participant
2. Row order is determined by sorting the participants using the expression values of the gene of interest (GOI)
3. Participants at extreme ends (Low and High) are grouped separately and subsequently compared

## 5.2 Composite gene analysis - Additive mode

Composite analyses use information from multiple genes simultaneously to divide individuals within a cohort population into distinct strata. The additive mode of TRANSECT uses expression information from multiple genes (2 – 5 genes in the current implementation) to rank individuals expressing each of the component genes at near to physiological extreme and separate them into low and high strata for subsequent DE analysis. This is achieved by computing the average of rank positions for all component genes for each participant and using the metric to position each individual within the cohort in order. Once this is achieved and in like manner to the single gene analysis, individuals with extreme high average rank positions are grouped and compared to individuals with extreme low rank positions.

It is important to note that this process leads, in most scenarios, to the exclusion of participants with extreme expression for any one (or more) of the component genes of interest. A good example of this can be seen from the additive mode case study for ESR1, PGR and ERBB2 (triple-negative breast cancer genes) in the RECOUNT3 BRCA cohort.

The figure below (a TRANSECT output for this type of analysis), plots the expression level separately for each component gene (here ESR1, PGR and ERBB2), for each participant. Expanding on this, each participant in the cohort occupies a single point on each of the three distributions in the figure, corresponding to the participants expression level for the three genes of interest. Featured on the figure below is participant TCGA-C8-A12Z-01A who possesses the highest expression level for ERBB2 ( $TPM=3747.42$ ) of all participants in the cohort however, does not express either ESR1 nor PGR ( $TPM=0.37$  and  $0.15$  respectively). Individuals stratified by TRANSECT who rank low for expression of all three receptors are marked in cyan (one point per individual on each of the three distributions) and conversely, those ranking high for elevated expression of all three receptor genes are marked in magenta.

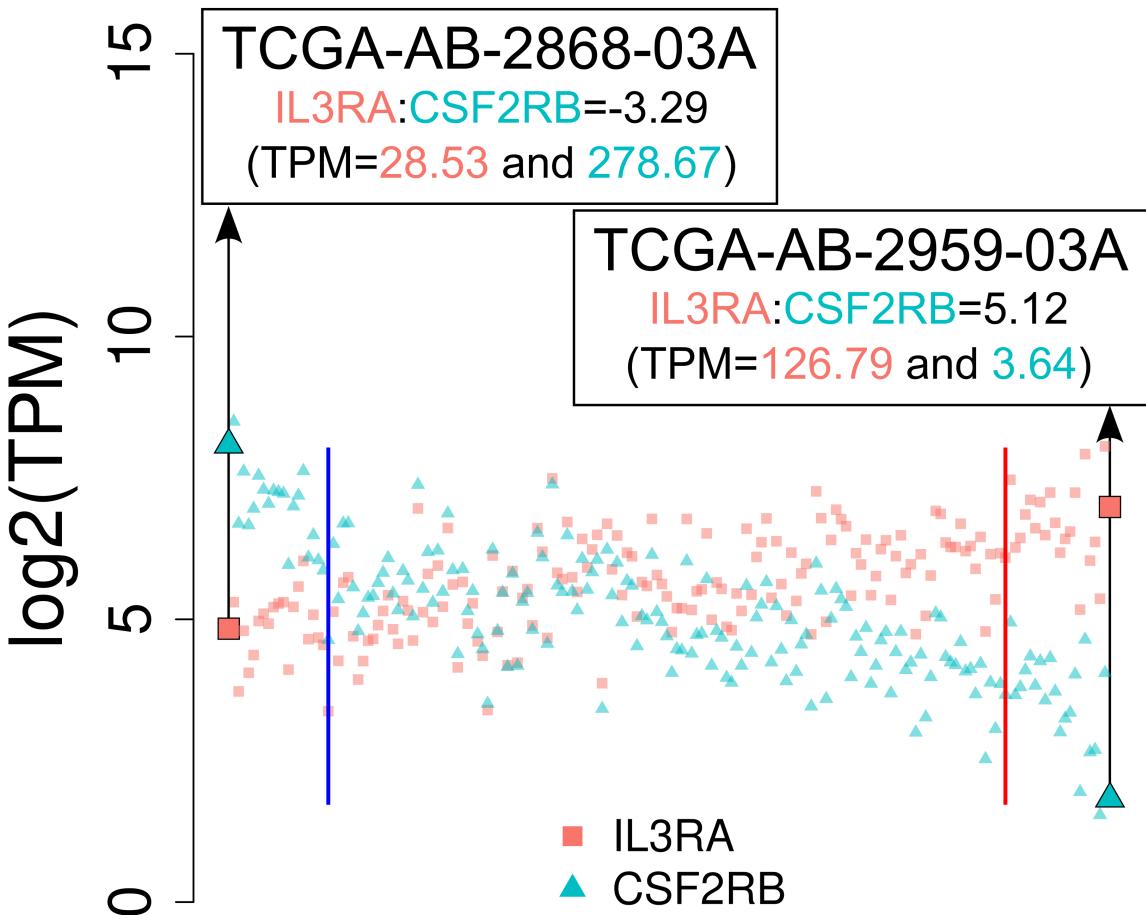


### 5.3 Composite gene analysis - Ratio mode

In the same manner as the additive mode described above, the ratio mode also considers information from multiple genes simultaneously to partition individuals within a cohort population into distinct strata. The ratio mode uses expression information from strictly two genes to rank individuals. In order to achieve this, TRANSECT calculates a simple log-ratio statistic (log fold change) between the 2 genes of interest for each patient and uses this as the rank metric. Extremely low ratio scores will demarcate participants where geneA >> geneB and vice versa. Again, individuals at both extremes are grouped and compared.

Like the additive mode, participants with extreme expression for any one of the component genes may, or may not, make it into the stratified groups for later comparison.

The figure below (another TRANSECT output for this type of analysis), plots the expression level separately for the two component genes (IL3RA and CSF2RB), for each participant. Expanding on this, here each participant in the cohort occupies two points on the plot (each point directly above and below the other), corresponding to the participants expression level for the two genes of interest. Participants are ordered across the x-axis from low to high based on their ratio score (converted to a rank metric). Featured on the figure below are two participants TCGA-AB-2868-03A and TCGA-AB-2959-03A who rank lowest and highest for this analysis in this cohort respectively, showing both the ratio score and TPM expression values. The solid vertical blue and red lines demarcate the thresholds for inclusion into the low and high strata respectively.



## Rank ordered participants

### 5.4 Multimodal analysis

In select large cohort studies there exist measurements derived from multiple omics for the same individual at the same or similar timepoints. For example, the TCGA study consists of RNA (mRNA, miRNA), and DNA (methylation, mutation, and copy number) data in addition to the associated global proteomics data generated by the Clinical Proteomic Tumor Analysis Consortium (CPTAC). TRANSECT has the facility to survey changes in one omics data type based on the stratification of individuals using matched data from another omics. As in the use cases above, individuals at each extreme are grouped and compared.

TRANSECT comes preconfigured with the ability to assess global changes in mRNAs based on the stratification of cohort participants based on their miRNA expression. This can only run whilst using GDC TCGA data that possess sufficient numbers of mRNA and miRNA expression data. Other types of multimodal analyses using different omics types require custom configuration.

## 6. Prepare commands and options

**Prepare** is a process that retrieves the raw data from online repositories and prepares it (if required) for analysis. TRANSECT comes bundled with three different prepare scripts, one each for RECOUNT3, GTEx and GDC-TCGA data.

All downloaded and formatted data is stored by default in the TRANSECT/data/<RECOUNT3|GTEx|GDC>/ subdirectory in individual folders named by tissue/cancer abbreviation. For example, the RECOUNT3 PRAD data downloaded in the basic demonstration of this manual is stored in /TRANSECT/data/RECOUNT3/PRAD/

## 6.1 RECOUNT3

Retrieve and prepare RECOUNT3 RNA-seq data for in-house custom analyses.

USAGE:

```
$ R3_prepare_directories.sh [-h] -p <RECOUNT3 Project ID>
```

PARAMETERS:

- h Show help text
- p RECOUNT3 project id: needs to be valid RECOUNT3 project id (ie. BRCA for TCGA data OR BREAST for GTEx). **Required**

You can retrieve and prepare more than one RECOUNT dataset by using a bash for loop like this;

```
$ for r3_code in COAD BREAST LAML; do R3_prepare_directories.sh -p $r3_code ; done
```

## 6.2 GTEx

Retrieve and prepare GTEx RNA-seq data for in-house custom analyses. Unlike RECOUNT3 and GDC data retrieval, GTEx data for all tissue types are retrieved in a single file. Subsequently, this is separated into tissue specific datasets using the information in the metadata file.

USAGE:

```
$ GTEx_prepare_directories.sh [-h] [-a -c -t]
```

PARAMETERS:

- h Show help text
- a retrieve all expression data (mRNA counts and TPMs), **Required** for the proper functioning of TRANSECT
- c retrieve only mRNA counts
- t retrieve only mRNA TPMs

## 6.3 GDC

Retrieve and prepare TCGA RNA-seq data for in-house custom analyses.

USAGE:

```
$ GDC_TCGA_prepare_directories.sh [-h] -p <TCGA Project ID> [-a -c -r -R -k -n]
```

## PARAMETERS:

- h** Show help text
- p** TCGA project id: needs to be valid TCGA project id as used by GDC (ie. TCGA-BRCA).

## Required

- a** retrieve all expression data (mRNA counts and TPMs as well as miR and isomiR RPMs)
- c** retrieve only mRNA counts
- r** retrieve only miR RPMs
- R** retrieve only isomiR RPMs
- k** keep all data (Default: False)
- n** data is not from TCGA study (Default: False)

To retrieve and prepare more than one TCGA cancer dataset use a bash for loop like this;

```
$ for tcga_code in TCGA-COAD TCGA-SARC TCGA-LAML; do  
GDC_TCGA_prepare_directories.sh -p $tcga_code; done
```

To retrieve and prepare all TCGA cancer datasets you can loop through all lines in GDC\_API/TCGA\_Study\_Abbreviations.tsv (WARNING: this requires lots of time, network and disc space)

```
$ while read tcga_code; do GDC_TCGA_prepare_directories.sh -p $tcga_code; done  
< <(cut -f1 GDC_API/TCGA_Study_Abbreviations.tsv | tail -n +2)
```

Please be aware that some of these collections are large and require substantial disk space. They can take a considerable amount of time to download and process. For example, downloading and processing GDC TCGA-BRCA takes just over 30 minutes (using a high speed network connection and an up to date workstation) and requires more than 14GB of disk space (most of which can and by default is, deleted afterwards). In comparison, GDC TCGA-LAML takes less than 5 minutes to retrieve and less than 2GB of disc space.

In addition, the GDC prepare script often fails when downloading large datasets. This is caused by network connectivity issues (tested only in Australia) with the GDC repository. If you experience issues, delete the relevant dataset and retry the prepare command.

## 7. Analysis commands and options

**Analyse** is a process that uses the prepared public data from above, conducts the stratified differential expression and produces all the outputs. Like with the prepare operations, TRANSECT comes bundled with three analyse scripts, one each for RECOUNT3, GTEx and GDC-TCGA.

Unlike the prepare operations, the output from these calls is saved in the current working directory and therefore it is recommended to create a descriptively named folder for each of your analyses. TRANSECT comes with an preinstalled output folder containing subdirectories (TRANSECT/output/<RECOUNT3|GTEx|GDC>/) however, you may choose any working directory at your discretion. Keep in mind that if TRANSECT output exists in the current working directory, it will be overwritten.

For each script, composite analyses can be run using the plus character (+) for additive combinations or by using the modulus character (%) for ratio. The two special characters are used between gene names like so. Additive example: ESR1+PGR+ERBB2 or Ratio example: ESRP1%ZEB1

## 7.1 RECOUNT3

Differential expression analysis of RECOUNT3 data stratified into high and low groups by gene of interest

Please run this wrapper script in the directory of the desired output location

USAGE:

```
$ R3_analyse_GOI.sh [-h] -p <RECOUNT3 projectID> -g <GOI> -s <StratifyBy> -t  
<Percentile> -e -S -a -c -d
```

PARAMETERS:

- h** Show help text
- p** RECOUNT3 tissue id: needs to be valid RECOUNT3 tissue id as at RECOUNT3 (ie. BRCA for TCGA or BREAST for GTEx). **Required**
- g** Gene of interest: needs to be a valid HGNC symbol (ie. ZEB1). **Required**
- s** Stratify by molecule: Must match -g and can only be mRNA at present. **Required**
- t** Percentile: stratify data into top and bottom x percentile (valid x between 2 and 25).

### Required

- e** Enrichment analyses: Run GSEA on DE results (Default: Only run WebGestalt)
- S** Switch pairwise comparison: find genes DE in low group compared to high group (Default: high compared to low)
- a** Do all analyses
- c** Do correlation analysis only
- d** Do differential expression analysis only

## 7.2 GTEx

Differential expression analysis of GTEx data stratified into high and low groups by gene of interest

Please run this wrapper script in the directory of the desired output location

USAGE:

```
$ GTEx_analyse_GOI.sh [-h] -p <GTExTissueID> -g <GOI> -s <StratifyBy> -t  
<Percentile> -e -S -a -c -d
```

PARAMETERS:

- h** Show help text
- p** GTEx tissue id: needs to be valid GTEx tissue id as at GTEx (ie. Breast). **Required**
- g** Gene of interest: needs to be a valid HGNC symbol (ie. ZEB1). **Required**
- s** Stratify by molecule: Must match -g and can only be mRNA at present. **Required**
- t** Percentile: stratify data into top and bottom x percentile (valid x between 2 and 25).

### Required

- e** Enrichment analyses: Run GSEA on DE results (Default: Only run WebGestalt)
- S** Switch pairwise comparison: find genes DE in low group compared to high group (Default: high compared to low)
- a** Do all analyses

- c Do correlation analysis only
- d Do differential expression analysis only

## 7.3 GDC

Differential expression analysis of TCGA data stratified into high and low groups by gene of interest

Please run this wrapper script in the directory of the desired output location

USAGE:

```
$ GDC_TCGA_analyse_GOI.sh [-h] -p <TCGAProjectID> -g <GOI> -s <StratifyBy> -t
<Percentile> -e -S -a -c -d
```

PARAMETERS:

- h Show this help text
- p TCGA project id: needs to be valid TCGA project id as at the GDC (ie. TCGA-BRCA). **Required**
- g Gene of interest: needs to be a valid HGNC symbol (ie. ZEB1). **Required**
- s Stratify by molecule: must match -g and can only be one of (mRNA or miRNA). **Required**
- t Percentile: stratify data into top and bottom x percentile (valid x between 2 and 25).

### Required

- e Enrichment analyses: Run GSEA on DE results (Default: Only run WebGestalt)
- S Switch pairwise comparison: find genes DE in low group compared to high group (Default: high compared to low)
  - a Do all analyses
  - c Do correlation analysis only
  - d Do differential expression analysis only

## 8. Output

---

TRANSECT takes in a cohort dataset and processes the data as follows.

1. First, TRANSECT partition the data by the expression of a gene/s of interest into low and high strata
2. Subsequently, TRANSECT compares the resulting strata, one to the other, to identify differentially expressed genes
3. And finally, TRANSECT uses the results from the DE analysis to run functional annotation and enrichment analyses

The outputs from TRANSECT are likewise grouped into 3 categories and returned in three folders in the working directory from where the program is executed

### 8.1 01-Stratification

The stratification process produces 2 tables, and 3 plots.

1. GOI\_exp\_raw\_OG.tsv contain the raw original expression (TPM) data for all gene/s of interest
2. GOI\_exp\_with\_strat.tsv contains the same data sorted with additional columns relating to the participants ranking score, percentiles and quantile values.

3. TPM\_histogram.html or TPM\_Boxplot\_Sina.html or TPM\_Scatter.html, all which plot data from the two tables above differently depending on the chosen TRANSECT mode in an attempt to describe the distribution of gene expression across the cohort participants
4. TPM\_N-T\_boxplot.html which shows the distribution of expression partitioned by disease state when available
5. TPM\_strat\_boxplot.html which plots the low and high strata participants resulting from the stratification process

## 8.2 02-DE

The DE analysis produces many tables and plots most easily described as follows.

1. DE Setup - design.tsv and gene\_raw\_expression\_data\_cpm.csv
2. DE QC - bcv and mean\_var.png plots as well as the MDS-Plot.html in the glimma-plots folder
3. Normalised expression tables - gene\_normalised\_expression\_data\_cpm.csv (also in log form)
4. DE result tables - High\_Vs\_Low\_de\_sigFC.csv and top\_tags.csv
5. DE result plots - High\_Vs\_Low\_volcano.png and High\_Vs\_Low\_heatmap.png as well as an interactive version of the volcano plot in the glimma-plots folder
6. The glimma-plots folder containing the interactive web plots and associated data

## 8.3 03-Enrichment

The 2 enrichment analyses result in the production of two folders each with a separate collection of tables and plots.

### 1. GSEA

When selected, this folder contains the output folders from running GSEA against the Hallmark as well as the Curated MSigDB collections respectively. Within each folder, users can open the index.html file to access and interact with the results in a web browser. In addition, the results are summarised and provided in tabular form (.csv) as well as interactive form (.html). See the [GSEA User Guide](#) for more details

GSEA input data – 3 text files used for the GSEA analysis are saved in the top-level folder. The default GSEA method used by TRANSECT is the pre-ranked method. Input for this analysis can be found in the .rnk file. Provided but not used by TRANSECT are alternate GSEA input files (.cls and .txt). These files can be used to rerun GSEA outside of TRANSECT with custom parameters against different collections.

### 2. WebGestalt

The ORA results are presented in six folders; two each for disease, gene ontology and pathway enrichment, for up and down regulated genes separately (when available). Within each folder, users can open the .html file to access and interact with the results in a web browser. See the [WebGestalt Manual](#) for more details

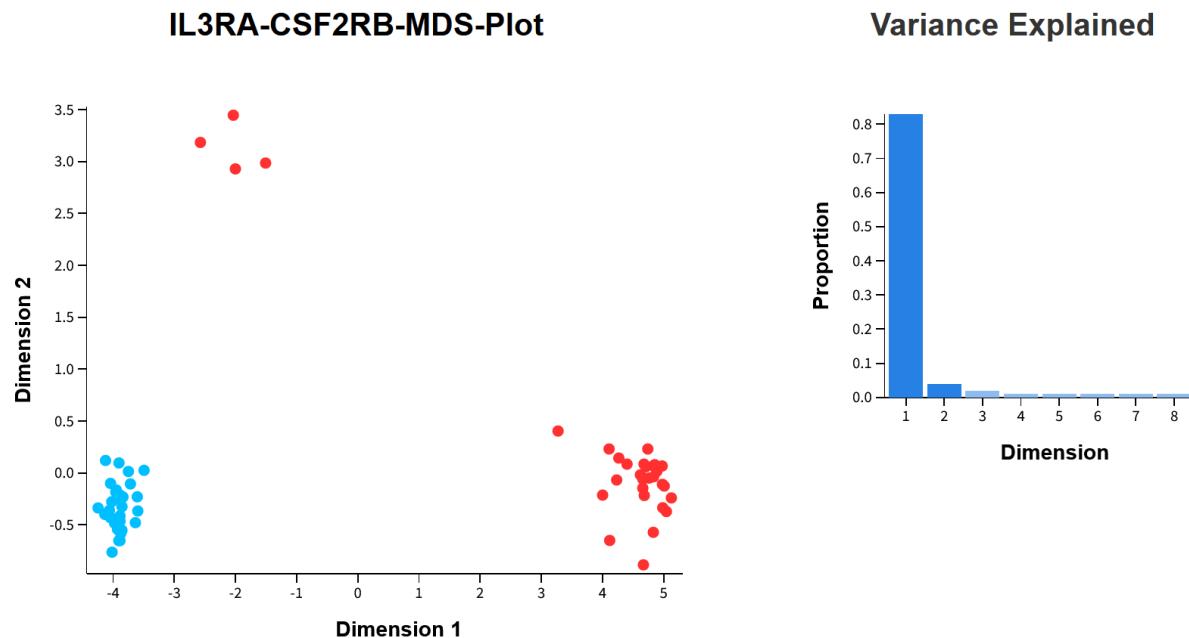
## 9. Precautions

### 9.1 Cohort size

TRANSECT requires large numbers of participants in the cohort data sets to adequately achieve appropriate stratification and grouping. Ideally, individual members of each stratum derived from the stratification process will share highly similar attributes or characteristics (here, gene expression levels). Cohort data sets with low participant numbers are unlikely to possess the required random sampling of a population to achieve defined stratum containing members with shared characteristics and may force the allocation of members with different characteristics into the same stratum.

### 9.2 Bulk RNA-seq heterogeneity

The heterogeneity of cell types within bulk tissue samples which are present in these cohort data sets can lead to misleading observations if not carefully considered. As an example, whilst examining one of our case studies using the RECOUNT3 GTEx Blood cohort (ratio mode - IL3RA%CSF2RB), we stumbled upon separation statistics in the MDS plot that appeared altogether unlikely and very dubious.



A few telling signs stand out. First, there is a huge near 90% explaining the separation between high (red) and low (blue) samples across dimension 1, something usually only observed between isogenic cell lines or technical replicates. Second, four samples from the high group appear **not** to belong? Diving into the GTEx metadata for the participants in these strata, we found that the RECOUNT3 GTEx Blood cohort had unaccounted substructure!

Below is the table of participants used in the above analysis separated by stratum. On the left is the low group (light blue background) with GTEx metadata showing consistently for each participant, membership in "GTEx SMTS Blood" and "GTEx SMTSD Whole Blood", as expected. On the right (light red background), the same for the high stratum showing that although all participants in this stratum are annotated as "GTEx SMTS Blood", the detailed annotation reveals all but four are actually "Cells - EBV-transformed lymphocytes". Unintentionally, we were comparing Whole Blood to EBV-transformed lymphocytes. (NOTE: "SMTS" - Tissue Type, area from which the tissue sample was taken and "SMTSD" - Tissue Type, more specific detail of tissue type)

GTEX ID (low stratum)	GTEX SMTS	GTEX SMTSD	GTEX ID (high stratum)	GTEX SMTS	GTEX SMTSD
GTEX-14H4A+C3:F63-0006-SM-5N9E3	Blood	Whole Blood	GTEX-UPJH-0001-SM-3NMDE	Blood	Cells - EBV-transformed lymphocytes
GTEX-11I78-0005-SM-5N9GB	Blood	Whole Blood	GTEX-VUSH-0004-SM-3P61T	Blood	Cells - EBV-transformed lymphocytes
GTEX-ZF29-0006-SM-4WKGQ	Blood	Whole Blood	GTEX-U412-0006-SM-3DB8J	Blood	Whole Blood
GTEX-14E6E-0006-SM-5MR5N	Blood	Whole Blood	GTEX-WCDI-0002-SM-3P61U	Blood	Cells - EBV-transformed lymphocytes
GTEX-1C475-0006-SM-9MQL7	Blood	Whole Blood	GTEX-1211K-0001-SM-5S2P9	Blood	Cells - EBV-transformed lymphocytes
GTEX-1QP29-0005-SM-DLZQX	Blood	Whole Blood	GTEX-XXEK-0004-SM-4BRW0	Blood	Cells - EBV-transformed lymphocytes
GTEX-13W3W-0005-SM-5Si9Y	Blood	Whole Blood	GTEX-1O97I-0005-SM-DKPPY	Blood	Whole Blood
GTEX-12BJ1-0006-SM-5SIB5	Blood	Whole Blood	GTEX-1GTWX-0003-SM-EYYVV	Blood	Cells - EBV-transformed lymphocytes
GTEX-UJMC-0005-SM-3GACU	Blood	Whole Blood	GTEX-13CF2-0006-SM-5O99L	Blood	Whole Blood
GTEX-1AX9J-0005-SM-9MQKU	Blood	Whole Blood	GTEX-SUCS-0002-SM-3NMAJ	Blood	Cells - EBV-transformed lymphocytes
GTEX-ZT9W-0005-SM-4YCEG	Blood	Whole Blood	GTEX-ZG7Y-0003-SM-4WWEJ	Blood	Cells - EBV-transformed lymphocytes
GTEX-YFCO-0005-SM-4W1ZI	Blood	Whole Blood	GTEX-1269C-0003-SM-5S2PB	Blood	Cells - EBV-transformed lymphocytes
GTEX-14PN3-0006-SM-7SB6O	Blood	Whole Blood	GTEX-1GMR2-0001-SM-EYYWA	Blood	Cells - EBV-transformed lymphocytes
GTEX-1FIGZ-0005-SM-A8N7O	Blood	Whole Blood	GTEX-1EU9M-0001-SM-EVR3A	Blood	Cells - EBV-transformed lymphocytes
GTEX-12WSN-0006-SM-5NQAP	Blood	Whole Blood	GTEX-11TT1-0004-SM-5S2NT	Blood	Cells - EBV-transformed lymphocytes
GTEX-1AMFI-0006-SM-9KNTQ	Blood	Whole Blood	GTEX-YFCO-0003-SM-4W21I	Blood	Cells - EBV-transformed lymphocytes
GTEX-SUCS-0006-SM-4DM59	Blood	Whole Blood	GTEX-1178-0001-SM-5Q5BE	Blood	Cells - EBV-transformed lymphocytes
GTEX-ZDTT-0006-SM-4WKFP	Blood	Whole Blood	GTEX-117XS-0005-SM-5PNU6	Blood	Whole Blood
GTEX-1KXAM-0005-SM-DIPEC	Blood	Whole Blood	GTEX-T6MN-0002-SM-5S2TI	Blood	Cells - EBV-transformed lymphocytes
GTEX-14DAR-0006-SM-5N9GC	Blood	Whole Blood	GTEX-1GMR8-0001-SM-EVR3E	Blood	Cells - EBV-transformed lymphocytes
GTEX-1122O-0005-SM-5O99J	Blood	Whole Blood	GTEX-RUSQ-0003-SM-2XCEB	Blood	Cells - EBV-transformed lymphocytes
GTEX-OHPL-0006-SM-3MJHB	Blood	Whole Blood	GTEX-1EW1Q-0001-SM-EVR3B	Blood	Cells - EBV-transformed lymphocytes
GTEX-1399U-0005-SM-5NQ8K	Blood	Whole Blood	GTEX-T6MN-0002-SM-3NMAH	Blood	Cells - EBV-transformed lymphocytes
GTEX-11EM3-0005-SM-5N9DK	Blood	Whole Blood	GTEX-V955-0004-SM-3NMDH	Blood	Cells - EBV-transformed lymphocytes
GTEX-P4PQ-0005-SM-2HMKJ	Blood	Whole Blood	GTEX-S32W-0004-SM-2XCE9	Blood	Cells - EBV-transformed lymphocytes
GTEX-1OKEX-0006-SM-DKPQ2	Blood	Whole Blood	GTEX-XUVS-0002-SM-47JXL	Blood	Cells - EBV-transformed lymphocytes
GTEX-15RIF-0005-SM-7RHGH	Blood	Whole Blood	GTEX-S32W-0004-SM-5S2SG	Blood	Cells - EBV-transformed lymphocytes
GTEX-1QP67-0006-SM-CY8H5	Blood	Whole Blood	GTEX-T5JC-0001-SM-5S2S4	Blood	Cells - EBV-transformed lymphocytes
GTEX-1JMPY-0006-SM-DHXJ8	Blood	Whole Blood	GTEX-T5JC-0001-SM-3NMAK	Blood	Cells - EBV-transformed lymphocytes
GTEX-S95S-0005-SM-2XCEC	Blood	Whole Blood	GTEX-RU1J-0003-SM-3K2A8	Blood	Cells - EBV-transformed lymphocytes
			GTEX-SN8G-0001-SM-3NML8	Blood	Cells - EBV-transformed lymphocytes

Undoubtedly, there is more substructure in the Whole Blood cohort, some subtle others maybe not?

## 10. Example commands

### Single gene analysis

```
#1. RECOUNT3 - BRCA, ZEB1 High Vs Low, stratification threshold of 5%, DE only
R3_analyse_GOI.sh -p BRCA -g ZEB1 -s mRNA -t 5 -d

#2. GTEX - Breast, ESR1 High Vs Low, stratification threshold of 7%, DE and
correlation analysis
GTEX_analyse_GOI.sh -p Breast -g ESR1 -s mRNA -t 7 -a

#3. GDC - TCGA-PRAD, AR Low Vs High (switch comparison), stratification
threshold of 3%, DE and correlation analysis, run GSEA
GDC_TCGA_analyse_GOI.sh -p TCGA-PRAD -g AR -s mRNA -t 3 -s -a -e
```

### Composite gene analysis - Additive mode

```
#1. RECOUNT3 - BREAST, ESR1+PGR+ERBB2 Low Vs High (switch comparison),
stratification threshold of 6%, DE only, run GSEA
R3_analyse_GOI.sh -p BREAST -g ESR1+PGR+ERBB2 -s mRNA -t 6 -s -d -e

#2. GDC - TCGA-KIRC, ZEB1+ESRP1 High Vs Low, stratification threshold of 10%,
DE and correlation analysis
GDC_TCGA_analyse_GOI.sh -p TCGA-KIRC -g ZEB1+ESRP1 -s mRNA -t 10 -a
```

### Composite gene analysis - Ratio mode

```
#1. RECOUNT3 - LAML, IL3RA-CSF2RB High Vs Low, stratification threshold of 12%,  
DE only, run GSEA  
R3_analyse_GOI.sh -p LAML -g IL3RA%CSF2RB -s mRNA -t 12 -d -e  
  
#2. GTEx - Breast, ESR1-PGR High Vs Low, stratification threshold of 2%, DE  
only, run GSEA  
GTEx_analyse_GOI.sh -p Breast -g ESR1%PGR -s mRNA -t 2 -d -e
```

## Multimodal analysis

```
#1. GDC - TCGA-BRCA, hsa-miR-200c-3p High Vs Low, stratification threshold of  
2%, DE only, run GSEA  
GDC_TCGA_analyse_GOI.sh -p TCGA-BRCA -g hsa-miR-200c-3p -s miRNA -t 2 -d -e
```

# 11. Publication details

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Currently unpublished.



This manual was produced in markdown using [typora](#) v1.9.5