Tutorial for running DeMixT and TmS

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In this tutorial, we use a subset of the bulk RNAseq data of prostate adenocarcinoma (PRAD) from TCGA (https://portal.gdc.cancer.gov/) as an example to demonstrate how to run `DeMixT`. The analysis pipeline consists of the following steps:

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- Obtaining raw read counts for the tumor and normal RNAseq data
- Loading libraries and data
- Data preprocessing
- Deconvolution using DeMixT

Obtain raw read counts for the tumor and normal RNAseq data

The raw read counts for the tumor and normal samples from TCGA PRAD are downloaded from TCGA data portal.

One can also generate the raw read counts from fastq or bam files by following the GDC mRNA Analysis Pipeline.

Load libraries and data

Load library

```
library(DeMixT)
```

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```
1 library(DeMixT)
```

Load input data

```
1 load("./docs/etc/PRAD.RData")
```

Three data are included in the `PRAD.RData`.

- `PRAD`: Read counts matrix (gene x sample) with genes as row names and sample ids as column names.
- `Normal.id`: TCGA ids of PRAD normal samples.
- `Tumor.id` TCGA ids of PRAD tumor samples.

A glimpse of PRAD:

```
head(PRAD[,1:5])
cat('Number of genes: ', dim(PRAD)[1], '\n')
cat('Number of normal sample: ', length(Normal.id), '\n')
cat('Number of tumor sample: ', length(Tumor.id), '\n')
```

Output:

```
         TCGA-CH-5761-11A TCGA-CH-5767-11B TCGA-EJ-7115-11A TCGA-EJ-7123-11A TCGA-EJ-7125-11A
TSPAN6               3876             7095             5542             2747             8465
TNMD                   14               51               13               24               63
DPM1                 1162             2665             1544             1974             2984
SCYL3                 777             1517             1096             1231             1514
C1orf112              136              343              214              280              339
FGR                   230              511              263              755              262
Number of genes:  59427
Number of normal sample:  20
Number of tumor sample:  30
```

Data preprocessing

Conduct data cleaning and normalization before running DeMixT.

```
PRAD = PRAD[, c(Normal.id, Tumor.id)]
selected.genes = 9000
cutoff_normal_range = c(0.1, 1.0)
cutoff_tumor_range = c(0, 2.5)
cutoff_step = 0.1
preprocessed_data = DeMixT_preprocessing(PRAD,
                                         Normal.id,
                                         Tumor.id,
                                         selected.genes,
                                         cutoff_normal_range,
                                         cutoff_tumor_range,
                                         cutoff_step)
PRAD_filter = preprocessed_data$count.matrix
sd_cutoff_normal = preprocessed_data$sd_cutoff_normal
sd_cutoff_tumor = preprocessed_data$sd_cutoff_tumor
```

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                                         Normal.id,
                                         Tumor.id,
                                         selected.genes,
                                         cutoff_normal_range,
                                         cutoff_tumor_range,
                                         cutoff_step)
PRAD_filter = preprocessed_data$count.matrix
sd_cutoff_normal = preprocessed_data$sd_cutoff_normal
sd_cutoff_tumor = preprocessed_data$sd_cutoff_tumor
cat("Normal sd cutoff:", preprocessed_data$sd_cutoff_normal, "\n")
cat("Tumor sd cutoff:", preprocessed_data$sd_cutoff_tumor, "\n")
cat('Number of genes after filtering: ', dim(PRAD_filter)[1], '\n')
```

Output:

```
Normal sd cutoff: 0.1 0.9
Tumor sd cutoff: 0 0.6
Number of genes after filtering:  9103
```

The function `DeMixT_preprocessing` identifies two intervals based on the standard deviation of the log-transformed gene expression for normal and tumor samples, respectively, within the pre-defined ranges (`cutoff_normal_range` and `cutoff_tumor_range`).

In this example, we choose to select about 9000 genes before running DeMixT with the GS (Gene Selection) method to ensure that our model-based gene selection maintains good statistical properties.

`DeMixT_preprocessing` outputs a list object `preprocessed_data` containing:

- `preprocessed_data$count.matrix`: Preprocesssed count matrix
- `preprocessed_data$sd_cutoff_normal`: Actual cut-off value when desired genes are selected for normal samples
- `preprocessed_data$sd_cutoff_tumor`: Actual cut-off value when desired genes are selected for tumor samples

Deconvolution using DeMixT

- To optimize the parameters in `DeMixT` for input data, we recommend testing an array of combinations of number of spike-ins and number of selected genes.
- The number of CPU cores used by the `DeMixT` function for parallel computing is specified by the parameter `nthread`. By default, `nthread = total_number_of_cores_on_the_machine - 1`. Users can adjust `nthread` to any number between 0 and the total number of cores available on the machine.
- For reference, `DeMixT` takes approximately 3-4 minutes to process the PRAD data in this tutorial for each parameter combination when `nthread` is set to 55.

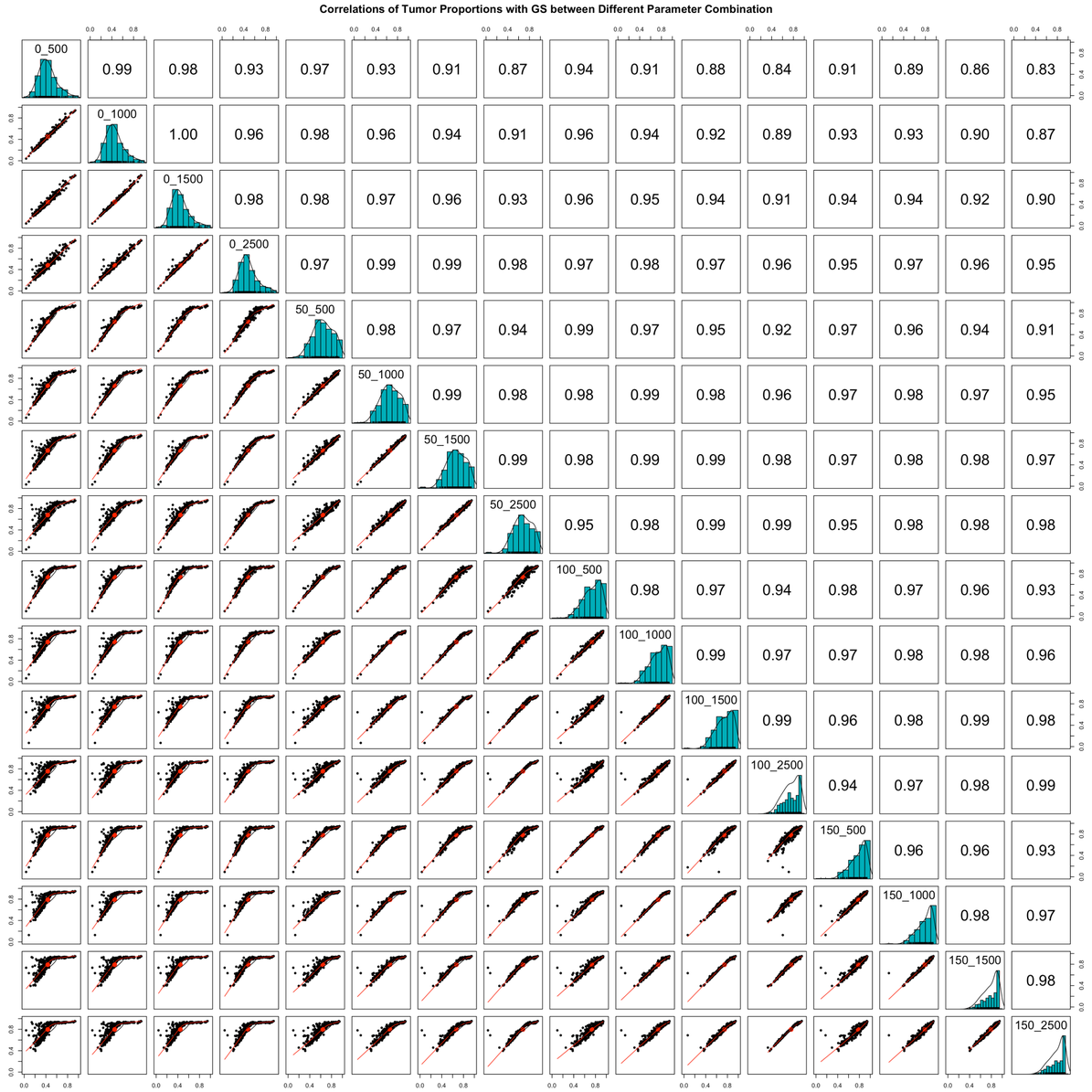
```r
# Due to the random initial values and the spike-in samples used in the DeMixT function,
# we recommand that users set seeds to ensure reproducibility.
# This seed setting will be incorporated internally in DeMixT in the next update.

set.seed(1234)

data.Y = SummarizedExperiment(assays = list(counts = PRAD_filter[, Tumor.id]))
data.N1 <- SummarizedExperiment(assays = list(counts = PRAD_filter[, Normal.id]))

# In practice, we set the maximum number of spike-in as min(n/3, 200),
# where n is the number of samples.
nspikesin_list = c(0, 5, 10)
# One may set a wider range than provided below for studies other than TCGA.
ngene.selected_list = c(500, 1000, 1500, 2500)

for(nspikesin in nspikesin_list){
    for(ngene.selected in ngene.selected_list){
        name = paste("PRAD_demixt_GS_res_nspikesin", nspikesin, "ngene.selected",
```

Note: We use a profiling likelihood-based method to select genes, during which we calculate confidence intervals for the model parameters using the inverse of the Hessian matrix. When the input data (e.g., gene expression levels from spatial transcriptomic data) is sparse, the Hessian matrix will contain infinite values, hence those confidence intervals can't be calculated. In this case, gene selection will be performed through differential expression analysis (identical to `DeMix_DE`). This alternative is automatically performed inside `DeMix_GS` when the above situation happens.

```r
PiT_GS_PRAD <- c()
row_names <- c()

for(nspikesin in nspikesin_list){
    for(ngene.selected in ngene.selected_list){
        name_simplify <- paste(nspikesin, ngene.selected,  sep = "_")
        row_names <- c(row_names, name_simplify)

        name = paste("PRAD_demixt_GS_res_nspikesin", nspikesin,
                     "ngene.selected", ngene.selected,  sep = "_");
        name = paste(name, ".RData", sep = "")
        load(name)
        PiT_GS_PRAD <- cbind(PiT_GS_PRAD, res$pi[2, ])
    }
}

colnames(PiT_GS_PRAD) <- row_names
```

This step saves the deconvolution results (PiT) into a dataframe with columns named after the combination of the number of spike-ins and number of genes selected. Then one can calculate and plot the pairwise correlations of estimated tumor proportions across different parameter combinations as shown in the next slide.

```r
pairs.panels(PiT_GS_PRAD,
             method = "spearman",
             # correlation method
             hist.col = "#00AFBB",
             density = TRUE,
             # show density plots
             ellipses = TRUE,
             # show correlation ellipses
             main = 'Correlations of Tumor
             Proportions with GS between
             Different Parameter Combination',
             xlim = c(0,1),
             ylim = c(0,1))
```



Print out the average pairwise correlation of tumor proportions across different parameter combinations.

```r
PiT_GS_PRAD <- as.data.frame(PiT_GS_PRAD)
Spearman_correlations <- list()

for(entry_1 in colnames(PiT_GS_PRAD)) {
  cor.values <- c()
  for (entry_2 in colnames(PiT_GS_PRAD)) {
    if (entry_1 == entry_2)
      next

    cor.values <- c(cor.values,
                    cor(PiT_GS_PRAD[, entry_1],
                    PiT_GS_PRAD[, entry_2],
                    method = "spearman"))
  }

  Spearman_correlations[[entry_1]] <- mean(cor.values)
}

Spearman_correlations <- unlist(Spearman_correlations)
```

```
num.spikein_num.selected.gene   mean.correlation
0_500            0.8641319
0_1000           0.9453534
0_1500           0.9401355
0_2500           0.9375468
5_500            0.9207604
5_1000           0.9542926
5_1500           0.9460006
5_2500           0.8992011
10_500           0.9237941
10_1000          0.9357266
10_1500          0.9249267
10_2500          0.9002124
```

We suggest selecting the optimal parameter combination that produces the `highest average correlation` of estimated tumor proportions.

Additionally, users are encouraged to evaluate the skewness of the PiT estimation distribution compared to a normal distribution centered around 0.5, as `Significant skewness may indicate biased estimation.`

num.spikein_num.selected.gene	mean.correlation
0_500	0.8641319
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0_1500	0.9401355
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5_2500	0.8992011
10_500	0.9237941
10_1000	0.9357266
10_1500	0.9249267
10_2500	0.9002124

Based on these criteria, `spike-ins = 5` and `number of selected genes = 1000` are identified as the optimal parameter combination. Using these parameters, we can obtain the corresponding tumor proportions.

```r
data.frame(sample.id=Tumor.id, PiT=PiT_GS_PRAD[['5_1000']])
```

```
sample.id                PiT
TCGA-2A-A8VL-01A    0.7596888
TCGA-2A-A8VO-01A    0.8421716
TCGA-2A-A8VT-01A    0.8662378
TCGA-2A-A8VV-01A    0.7616749
TCGA-2A-A8W1-01A    0.8291091
TCGA-2A-A8W3-01A    0.8159406
TCGA-CH-5737-01A    0.7314935
TCGA-CH-5738-01A    0.4614545
TCGA-CH-5739-01A    0.6349423
TCGA-CH-5740-01A    0.7095117
```

List the tumor specific expression

```r
## Load the corresponding deconvolved gene expression
load("PRAD_demixt_GS_res_nspikesin_5_ngene.selected_1000.RData")
res$ExprT[1:5, 1:5]
```

```
      TCGA-2A-A8VL-01A TCGA-2A-A8VO-01A TCGA-2A-A8VT-01A TCGA-2A-A8VV-01A TCGA-2A-A8W1-01A
DPM1          1710.194         1466.484        1680.4562         1644.944         1812.600
FUCA2         3782.990         4083.382         961.0578         4165.612         1896.901
GCLC          2382.106         1826.957        1527.4895         1409.707         1913.784
LAS1L         3329.766         2758.414        3520.9410         2834.415         2530.621
ENPP4         2099.591         3123.365        3173.3516         2856.371         7413.330
```

Instead of selecting using the parameter combination with the highest correlation, one can also select the parameter combination that produces estimated tumor proportions that are most biologically meaningful.

Next,

We will provide a simple TmS tutorial which uses The estimated tumor-specific proportions (PiT) genertated from DeMixT. For more details, visit https://wwylab.github.io/TmS/articles/TmS.html.

TmS Calcualtion

Tumor-specific total mRNA expression (TmS) from bulk sequencing data, taking into account tumor transcript proportion, purity and ploidy, which are estimable through transcriptomic/genomic deconvolution.

TmS analysis pipeline consists of the following steps:

- **Step 1**: Estimate the proportion of total RNA expression (π) from tumor cells using RNAseq data.
  - Achieved by using `DeMixT`[1]
- **Step 2**: Estiamte the proportion of tumor cells and total copies of haploid genomes, i.e., tumor purity (ρ) and tumor ploidy (ψ), using matched DNAseq or SNP array data.
- **Step 3**: Calculate TmS, the per cell haploid genome total RNA expression for tumor, using the estimated (π), (ρ) and (ψ): $TmS = [\pi(1-\rho)2]/[(1-\pi)\rho\psi]$.

Step 3: Calculate TmS using the estimated (π), (ρ) and (ψ).

Consensus TmS estimation

For DNA-based deconvolution methods such as ASCAT and ABSOLUTE, there could be multiple tumor purity ρ and ploidy ψ pairs that have similar likelihoods. Both ASCAT and ABSOLUTE can accurately estimate the product of purity ρ and ploidy ψ; however, they sometimes lack power to identify and separately. TmS is derived from the product of tumor ploidy and the odds of tumor purity. Hence, it is potentially more robust to ambiguity in the tumor purity and ploidy estimation, ensuring the robustness of the TmS calculation.

To calculate one final set of TmS values for a maximum number of samples, we use a consensus approach. We first calculate TmS values with tumor purity and ploidy estimates derived from both ABSOLUTE and ASCAT, and then fit a linear regression model on the log2-transformed TmS_{ASCAT} using the log2-transformed $TmS_{ABSOLUTE}$ as a predictor variable. We remove samples with Cook's distance ≥ 4/n and calculate the final

$$TmS = \sqrt{TmS_{ASCAT} \times TmS_{ABSOLUTE}}$$

The agreement between the two methods in ploidy values was low in 20% of TCGA samples. However, a large portion of these samples showed consistency in the TmS values using either ASCAT and ABSOLUTE, reducing the number of filtered TCGA samples to ~5% (264 samples) [2]. This result supports the robustness of our consensus approach.

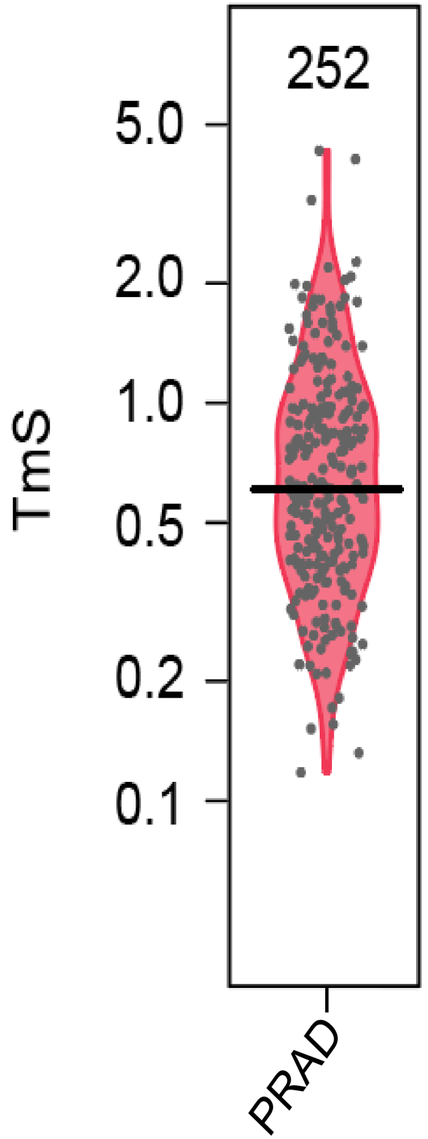
Input: Tumor-specific total mRNA proportions, tumor purities, tumor ploidies

Output: Consensus TmS

- `p`: Tumor-specific total mRNA proportions estimated by DeMixT
- `rho_ASCAT`: tumor purity estimated by ASCAT
- `phi_ASCAT`: tumor ploidy estimated by ASCAT
- `rho_ABSOLUTE`: tumor purity estimated by ABSOLUTE
- `phi_ABSOLUTE`: tumor ploidy estimated by ABSOLUTE

```r
TmS.calculate = function(p, rho, phi){
  return(2 * p * (1 - rho) / (phi * rho * (1 - p)))
}

TmS.ASCAT = TmS.calculate(p, rho_ASCAT, phi_ASCAT)
TmS.ABSOLUTE = TmS.calculate(p, rho_ABSOLUTE, phi_ABSOLUTE)

TmS.df = data.frame(TmS.ASCAT.log2 = log2(TmS.ASCAT),
                    TmS.ABSOLUTE.log2 = log2(TmS.ABSOLUTE))

lm.fit = lm(TmS.df$TmS.ABSOLUTE.log2 ~ TmS.df$TmS.ASCAT.log2)
summary(lm.fit)
cooksd = cooks.distance(lm.fit)
cooksd.threshold = 4 / nrow(TmS.df)

cook.status = rep("Accept", nrow(TmS.df))
cook.status[cooksd > cooksd.threshold] = "Outlier"
TmS.df = TmS.df[cook.status == "Accept", ]
```

The estimated TmS values for TCGA PRAD tumor samples are shown in the violin plot below.



Reference

[1] https://github.com/wwylab/DeMixT

[2] Cao, S. et al. Estimation of tumor cell total mRNA expression in 15 cancer types predicts disease progression. Nat Biotechnol (2022). https://doi.org/10.1038/s41587-022-01342-x.