DESeq2-normalized counts: Median of ratios method

Since tools for differential expression analysis are comparing the counts between sample groups for the same gene, gene length does not need to be accounted for by the tool. However, **sequencing depth** and **RNA composition** do need to be taken into account.

To normalize for sequencing depth and RNA composition, DESeq2 uses the median of ratios method. On the user-end there is only one step, but on the back-end there are multiple steps involved, as described below.

NOTE: The steps below describe in detail some of the steps performed by DESeq2 when you run a single function to get DE genes. Basically, for a typical RNA-seq analysis, **you would not run these steps individually**.

Step 1: creates a pseudo-reference sample (row-wise geometric mean)

For each gene, a pseudo-reference sample is created that is equal to the geometric mean across all samples.

gene	sampleA	sampleB	pseudo-reference sample
EF2A	1489	906	sqrt(1489 * 906) = **1161.5**
ABCD1	22	13	sqrt(22 * 13) = **17.7**
...	...	...	...

Step 2: calculates ratio of each sample to the reference

For every gene in a sample, the ratios (sample/ref) are calculated (as shown below). This is performed for each sample in the dataset. Since the majority of genes are not differentially expressed, the majority of genes in each sample should have similar ratios within the sample.

gene	sampleA	sampleB	pseudo-reference sample	ratio of sampleA/ref	ratio of sampleB/ref
EF2A	1489	906	1161.5	1489/1161.5 = **1.28**	906/1161.5 = **0.78**
ABCD1	22	13	16.9	22/16.9 = **1.30**	13/16.9 = **0.77**

gene	sampleA	sampleB	pseudo-reference sample	ratio of sampleA/ref	ratio of sampleB/ref
MEFV	793	410	570.2	793/570.2 = **1.39**	410/570.2 = **0.72**
BAG1	76	42	56.5	76/56.5 = **1.35**	42/56.5 = **0.74**
MOV10	521	1196	883.7	521/883.7 = **0.590**	1196/883.7 = **1.35**
...	...	...	...		

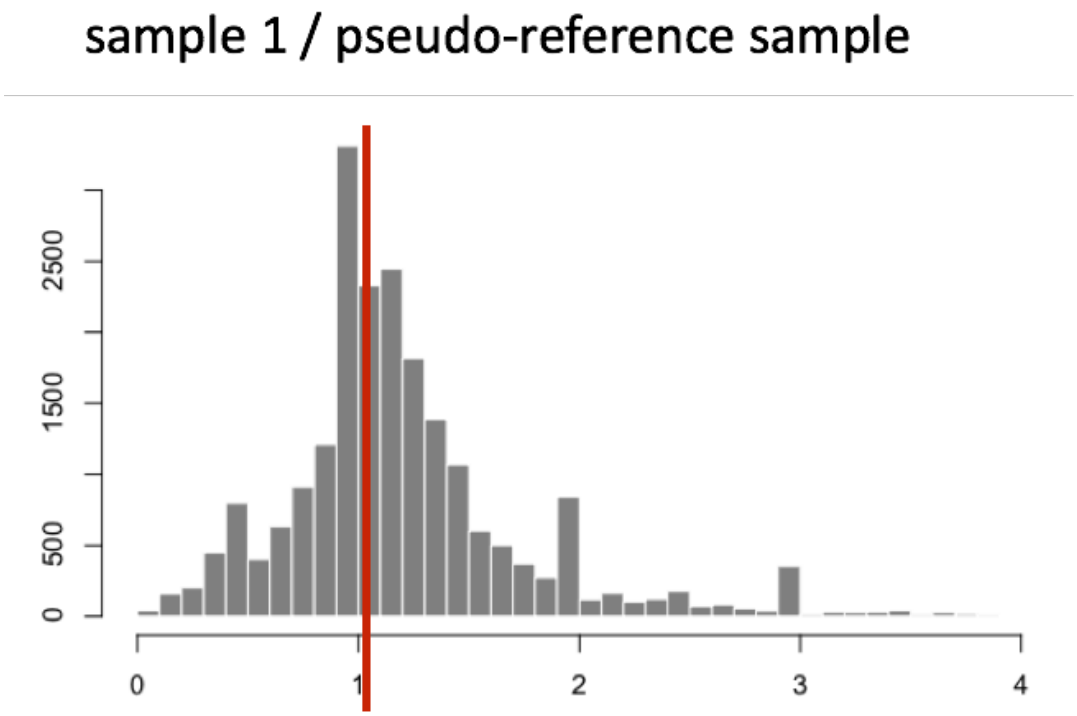
Step 3: calculate the normalization factor for each sample (size factor)

The median value (column-wise for the above table) of all ratios for a given sample is taken as the normalization factor (size factor) for that sample, as calculated below. Notice that the differentially expressed genes should not affect the median value:

```
normalization_factor_sampleA <- median(c(1.28, 1.3, 1.39, 1.35, 0.59))
```

```
normalization_factor_sampleB <- median(c(0.78, 0.77, 0.72, 0.74, 1.35))
```

The figure below illustrates the median value for the distribution of all gene ratios for a single sample (frequency is on the y-axis).



The median of ratios method makes the assumption that not ALL genes are differentially expressed; therefore, the normalization factors should account for sequencing depth and RNA composition of the sample (large outlier genes will not represent the median ratio values). **This method is robust to imbalance in up-/down-regulation and large numbers of differentially expressed genes.**

Usually these size factors are around 1, if you see large variations between samples it is important to take note since it might indicate the presence of extreme outliers.

Step 4: calculate the normalized count values using the normalization factor

This is performed by dividing each raw count value in a given sample by that sample's normalization factor to generate normalized count values. This is performed for all count values (every gene in every sample). For example, if the median ratio for SampleA was 1.3 and the median ratio for SampleB was 0.77, you could calculate normalized counts as follows:

SampleA median ratio = 1.3

SampleB median ratio = 0.77

Raw Counts

gene	sampleA	sampleB
EF2A	1489	906
ABCD1	22	13
...	...	...

Normalized Counts

gene	sampleA	sampleB
EF2A	1489 / 1.3 = **1145.39**	906 / 0.77 = **1176.62**

gene	sampleA	sampleB
ABCD1	22 / 1.3 = **16.92**	13 / 0.77 = **16.88**
...	...	...

Please note that normalized count values are not whole numbers.

Ref: https://hbctraining.github.io/DGE_workshop/lessons/02_DGE_count_normalization.html