OTU-Table Normalization Script

Task

Compensate for differential sequencing depth between samples by calculating relative abundances and create normalized counts. Use rarefaction curves to help estimate the sufficiency of sequencing depth for each sample.

Background

Rarefaction curves are useful for estimating the sufficiency of sequencing depth/effort per sample. This is achieved by observing how the number of species (richness) change over increasing number of reads. If the terminal slope of the curve levels, meaning the number of species/OTUs plateaus, then the sequencing effort in that sample was sufficient and additional sequencing will not bring additional knowledge on the diversity of the sample. If the slope remains steep even after all reads available were assigned then it indicates that additional sequencing was needed.

Normalization is the process of data transformation to remove the effect of differential sampling size. Because high-throughput sequencing results in different number of sequences per samples, a normalization of the read counts is required prior to downstream analysis. This is commonly performed by a procedure called rarefying, a random sub-sampling of reads from each sample to a fixed total, usually the least count among the samples. The process is repeated several times and a mean number of sequences is calculated for each OTU within the given sample. These values are then rounded and represent the final normalized counts. Rarefaction, although very popular among ecologists and microbial ecologists, has been criticized for the following reasons: (i) omission of available valid data, (ii) the estimation of overdispersion is more difficult due to data loss, (iii) loss of power (type II error), (iv) dependence on an arbitrary threshold, (v) additional uncertainty due to the randomness in rarefaction [1]. The authors of the latter publication stated that even a simple normalization to proportions is less biased as it includes no random steps and minimal loss of information. Their suggested normalization consisted of a variance stabilization transformation (logarithmic). In Rhea, the issue with this kind of transformation is the incompatibility with some of the downstream analytical functions requiring counts or proportions. Plotting of relative abundances across groups expressed in percentages, with its known limitations, gives researchers a more intuitive understanding of biological phenomenon. Hence, in Rhea, counts are by standard normalized via simple division to their sample size and then multiplication by the size of the smaller sample. This approach has not the downside of introducing random variance or loss of data. Nevertheless, we provide to users the option to proceed with classical rarefaction for normalized counts if wanted.

References

1. McMurdie, P. J., & Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput Biol*, *10*(4), e1003531.

Input

The expected input file for the script is a standard OTU-table (as tab-delimited text format) created by programs such as IMNGS (www.imngs.org). OTU names are in the first column followed by the count of reads assigned to each OTU cluster for each sample, with the sample name being the header of each column. The last column of the table must be named "taxonomy" and contains the taxonomic classification of each OTU. For compliance with downstream analysis, the taxonomy string for each OTU must be delimited by semicolons, with exactly 6 fields (left empty if not known).

#OTUId	Sample1	Sample2	Sample3	Sample4	taxonomy
OTU_1	1871	1820	2745	4952	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;;
OTU_2	2414	367	2056	1215	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;Alistipes;
OTU_3	236	269	88	244	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;;
OTU_4	224	432	196	318	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Clostridium XlVb;
OTU_5	376	68	144	27	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_6	1304	25	362	339	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides;
OTU_7	209	804	36	18	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_8	270	108	152	44	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_9	192	24	93	147	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_10	322	53	1071	573	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides;

Output

The script produces 6 output files. The first 4 containing either relative abundances with or without taxonomy, or normalized counts calculated from the relative abundances with or without taxonomy. If the folder structure of Rhea is preserved, copies of the output files are placed wherever needed for downstream analyses using the appropriate other scripts. The other 2 files are for estimating the sufficiency of sequencing depth. One is the pdf plot of rarefaction curves for all samples and the top under sequenced samples. The other is a tab delimited file with the terminal slope for each sample. The slope is calculated as number of species per 100 sequences.

For the template input table shown above, the output tables would be:

Relative abundance

	Sample1	Sample2	Sample3	Sample4
OTU_1	25.22243	45.84383	39.53622	62.86657
OTU_2	32.54246	9.244332	29.61256	15.42465
OTU_3	3.181451	6.775819	1.267464	3.097626
OTU_4	3.019682	10.88161	2.822987	4.03707
OTU_5	5.068752	1.712846	2.074031	0.34277
OTU_6	17.57886	0.629723	5.213884	4.303669
OTU_7	2.817471	20.25189	0.518508	0.228513
OTU_8	3.639795	2.720403	2.189255	0.558588
OTU_9	2.588299	0.604534	1.339479	1.866193
OTU_10	4.340793	1.335013	15.42561	7.274343

Relative abundance with taxonomy

	Sample1	Sample2	Sample3	Sample4	taxonomy
OTU_1	25.22243	45.84383	39.53622	62.86657	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;;
OTU_2	32.54246	9.244332	29.61256	15.42465	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;Alistipes;
OTU_3	3.181451	6.775819	1.267464	3.097626	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;;
OTU_4	3.019682	10.88161	2.822987	4.03707	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Clostridium XlVb;
OTU_5	5.068752	1.712846	2.074031	0.34277	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_6	17.57886	0.629723	5.213884	4.303669	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides;
OTU_7	2.817471	20.25189	0.518508	0.228513	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_8	3.639795	2.720403	2.189255	0.558588	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_9	2.588299	0.604534	1.339479	1.866193	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_10	4.340793	1.335013	15.42561	7.274343	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides;

Normalized counts

	Sample1	Sample2	Sample3	Sample4
OTU_1	1001.331	1820	1569.588	2495.803
OTU_2	1291.936	367	1175.619	612.3588
OTU_3	126.3036	269	50.31831	122.9758
OTU_4	119.8814	432	112.0726	160.2717
OTU_5	201.2294	68	82.33905	13.60797
OTU_6	697.8808	25	206.9912	170.8557
OTU_7	111.8536	804	20.58476	9.071982
OTU_8	144.4999	108	86.91344	22.17596
OTU_9	102.7555	24	53.1773	74.08785
OTU_10	172.3295	53	612.3967	288.7914

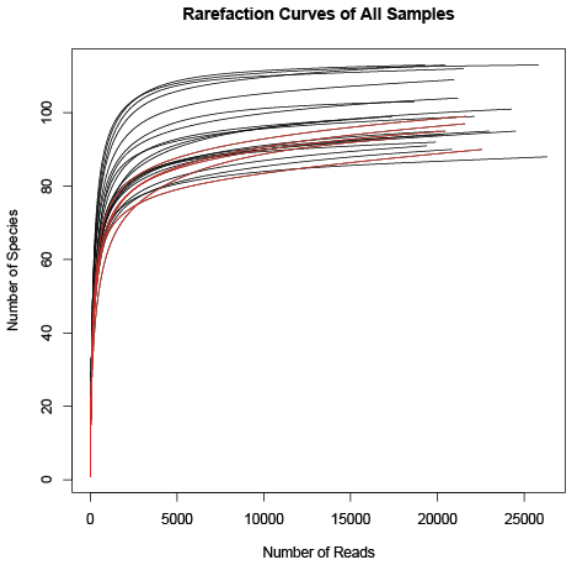
Normalized counts with taxonomy

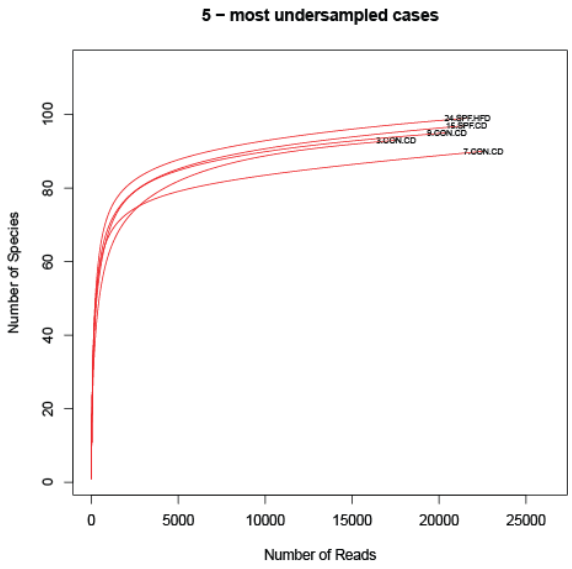
	Sample1	Sample2	Sample3	Sample4	taxonomy
OTU_1	1001.331	1820	1569.588	2495.803	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;;
OTU_2	1291.936	367	1175.619	612.3588	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;Alistipes;
OTU_3	126.3036	269	50.31831	122.9758	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;;
OTU_4	119.8814	432	112.0726	160.2717	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Clostridium XlVb;
OTU_5	201.2294	68	82.33905	13.60797	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_6	697.8808	25	206.9912	170.8557	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides;
OTU_7	111.8536	804	20.58476	9.071982	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_8	144.4999	108	86.91344	22.17596	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;;
OTU_9	102.7555	24	53.1773	74.08785	Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfovibrionaceae;;
OTU_10	172.3295	53	612.3967	288.7914	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides;

Rarefaction curves terminal slopes

SampleID	slope
7.CON.CD	0.89104925
15.SPF.CD	0.858093684
3.CON.CD	0.845982896
24.SPF.HFD	0.779135627
9.CON.CD	0.718256282
19.SPF.HFD	0.69613361
16.SPF.CD	0.648409594
22.SPF.HFD	0.611098764
14.SPF.CD	0.610023057
11.CON.HFD	0.574959357

Rarefaction Curve plots





Important Notes

Normalization is very sensitive to grossly different sample sizes. If for example a negative control sample is included in the OTU-table, all the samples would be normalized to the total number of reads in this control sample (which is likely very low). This would result in grossly wrong normalized counts and ensuing estimations of *alpha*- and *beta*-diversity. To avoid such errors, samples characterized by total read counts that fall out of the range of the majority of samples should be removed. These not only relates to negative controls, but also to samples to which low number of sequences were assigned due to various technical reasons. To help identify and judge the sufficiency of sequencing and normalization depth, a rarefaction curve is plotted for each sample and presented to the user. In addition, a selection of top (default 5) samples with the steepest curves are shown in separate plot to enhance the view of the most problematic samples. The steepness represents the level of saturation due to the depth of sequencing in terms of discovery of new species. It is expected that users have a very close look at original input tables and identify samples to be removed. If IMNGS is used for generation of the OTU-table, re-processing of the raw reads using a modified mapping file that includes only the selected sample-barcode combinations is required.

Common problems

- The path to the script is not set correctly
- The input file name is incorrect
- The input file is of a different format (e.g. has wrong taxonomic classification column)