

# OptiFit: a fast method for fitting amplicon sequences to existing OTUs

2021-06-23

Kelly L. Sovacool<sup>1</sup>, Sarah L. Westcott<sup>2</sup>, M. Brodie Mumphrey<sup>1</sup>, Gabrielle A. Dotson<sup>1</sup>,  
Patrick D. Schloss<sup>2†</sup>

<sup>1</sup> Department of Computational Medicine and Bioinformatics, University of Michigan

<sup>2</sup> Department of Microbiology and Immunology, University of Michigan

† To whom correspondence should be addressed: [pschloss@umich.edu](mailto:pschloss@umich.edu)

## Abstract

Assigning amplicon sequences to Operational Taxonomic Units (OTUs) is an important step in characterizing the composition of microbial communities across large datasets. OptiClust, a *de novo* OTU clustering method in the mothur program, has been shown to produce higher quality OTU assignments than other methods and at comparable or faster speeds (1, 2). A notable difference between *de novo* clustering and database-dependent methods is that OTU assignments clustered with *de novo* methods are not stable when new sequences are added to a dataset (3). However, in some cases one may wish to incorporate new samples into a previously clustered dataset without performing clustering again on all sequences, such as when deploying a machine learning model where OTUs are features (4). To provide an efficient and robust method to fit amplicon sequence data to existing OTUs, we developed the OptiFit algorithm as a new component of the mothur program. We tested OptiFit using four microbiome datasets with two different strategies; by fitting to an external reference database, or by splitting the dataset into a reference and query set and fitting the query sequences to the reference set. OptiFit produces OTUs of similar quality as OptiClust and at faster speeds when using the split dataset strategy, while the OTU quality and speed depends on the database chosen when using the external database strategy. OptiFit provides a suitable option for users who require stable reference-based OTU assignments at the same quality afforded by *de novo* clustering methods.

## Importance

## TODO

## Introduction

Amplicon sequencing has become a mainstay of microbial ecology and host-associated microbiome research. Researchers can affordably generate millions of sequences to characterize the composition of hundreds of samples from culture-independent microbial communities. In a typical analysis pipeline, 16S rRNA gene sequences are assigned to Operational Taxonomic Units (OTUs) to facilitate comparison of taxonomic composition between communities. A distance threshold of 3% (or sequence similarity of 97%) is commonly used to cluster sequences into OTUs based on either a reference database or pairwise comparisons of the sequences within the dataset. The method chosen for clustering affects the quality of OTU assignments and thus may impact downstream analyses of community composition (1, 3, 5).

There are two main categories of OTU clustering algorithms: *de novo* and reference-based. OptiClust, the *de novo* clustering method provided by the mothur software program, uses the distance score between all pairs of sequences in the dataset to cluster them into OTUs by maximizing the Matthews correlation coefficient (MCC) (1). This approach takes into account the distances between all pairs of sequences when assigning query sequences to OTUs, in contrast to other tools such as VSEARCH which only considers the distance between the query sequence and a representative centroid sequence in the OTU. A limitation of *de novo* clustering is that different OTU assignments will be produced when new sequences are added to a dataset, making it difficult to use *de novo* clustering to compare OTUs between different studies. Furthermore, since *de novo* clustering requires distance calculations between all sequences in a dataset, the execution time can be slow for very large datasets.

Reference clustering attempts to overcome the limitations of *de novo* clustering methods by using a representative set of sequences from a database, with each reference sequence seeding an OTU. Commonly, the Greengenes set of representative full length sequences

clustered at 97% similarity is used as the reference with VSEARCH (6). Query sequences are then assigned to OTUs based on their similarity to the reference sequences. Any query sequences that are not within the distance threshold to any of the reference sequences are either thrown out (closed reference mode) or clustered *de novo* to create additional OTUs (open reference mode). While reference-based clustering is generally fast, it is limited by the diversity of the reference database. Rare or novel sequences in the sample will be lost in closed reference mode if they are not represented by a similar sequence in the database. Previous studies found that the OptiClust *de novo* clustering algorithm created the highest quality OTU assignments of all clustering methods based on the Matthews correlation coefficient (MCC) (1). As a result, we have recommended OptiClust as the preferred method for OTU clustering whenever OTU stability is not required.

To overcome the limitations of *de novo* clustering while maintaining OTU quality, we developed OptiFit, a reference-based clustering algorithm in the mothur program which takes existing OTUs as the reference to fit new sequences to. In contrast to other tools, OptiFit considers all pairwise distance scores between reference and query sequences when assigning sequences to OTUs in order to produce OTUs of the highest possible quality. Here, we tested the OptiFit algorithm with the reference as a database or *de novo* OTUs and compared the performance to existing tools. To evaluate the OptiFit algorithm and compare to existing methods, we used four published datasets isolated from soil (7), marine (8), mouse gut (9), and human gut (10) samples.

## Results

### The OptiFit algorithm

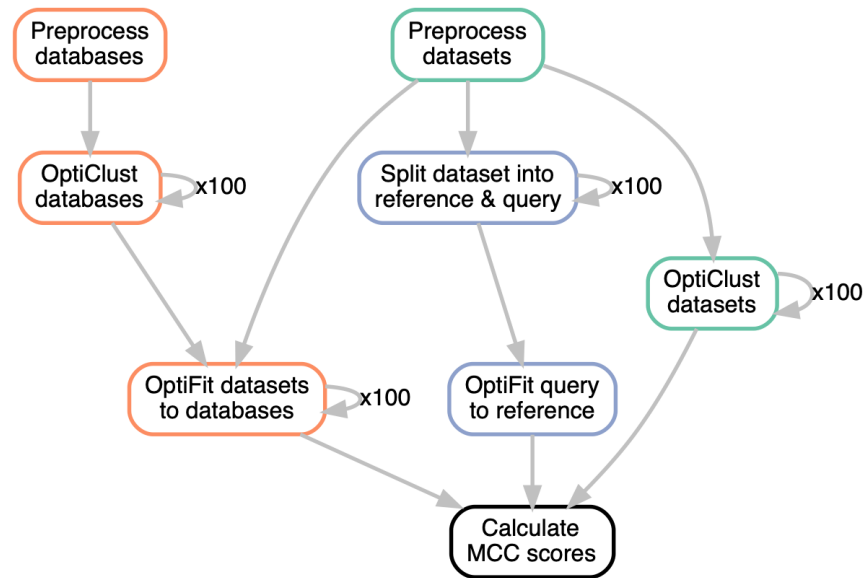
OptiFit leverages the method employed by OptiClust of iteratively assigning sequences to OTUs to produce the highest quality OTUs possible, and extends this method for reference-based clustering. OptiClust first seeds each sequence into its own OTU as a

singleton. Then for each sequence, OptiClust considers whether the sequence should move to a different OTU or remain in its current OTU, choosing the option that results in a better Matthews correlation coefficient (MCC). Iterations continue until the MCC stabilizes or until a maximum number of iterations is reached. This process produces *de novo* OTU assignments with the most optimal MCC given the input sequences. OptiFit begins where OptiClust ends, starting with a list of reference OTUs and their sequences, a list of query sequences to assign to the reference OTUs, and the sequence pairs that are within the distance threshold (e.g. 0.03). Initially, query sequences are placed in singleton OTUs. Then, the algorithm iteratively reassigns the query sequences to the reference OTUs to optimize the MCC. Alternatively, a sequence will remain unassigned if the MCC value is maximized when the sequence is a singleton rather than assigned to a reference OTU. This process is repeated until the MCC changes by no more than 0.0001 (default) or until a maximum number of iterations is reached (default: 100). In the closed reference mode, any query sequences that cannot be assigned to reference OTUs are discarded, and the results will only contain OTUs that exist in the original reference. In the open reference mode, unassigned query sequences are clustered *de novo* using OptiClust to generate new OTUs. The final MCC is reported with the best OTU assignments. There are two strategies for generating OTUs with OptiFit: 1) fit query sequences to reference OTUs generated by *de novo* clustering an independent database, or 2) split the dataset into a reference and query fraction, cluster the reference sequences *de novo*, then fit the query sequences to the reference OTUs. We clustered sequences from four datasets isolated from soil (7), marine (8), mouse gut (9), and human gut (10) samples to test the performance of OptiFit with both of these strategies.

## **Reference clustering with public databases**

While *de novo* clustering produces high quality OTUs, researchers may prefer to perform reference clustering to a public database because reference-based methods produce

100 consistent OTUs and are generally faster than *de novo* methods. In closed reference  
 101 mode, sequences that cannot be assigned to reference OTUs are thrown out, so that  
 102 the final clustering contains only OTUs that exist in the reference. To test how OptiFit  
 103 performs for this purpose, we fit each dataset to three databases of reference OTUs: the  
 104 Greengenes database, the SILVA non-redundant database, and the Ribosomal Database  
 105 Project (RDP) (6, 11, 12). Reference OTUs for each database were created by performing  
 106 *de novo* clustering with OptiClust at a distance threshold of 3% (see Figure 1). The *de*  
 107 *nov*o MCC scores were 0.72, 0.74, and 0.73 for gg, rdp, and silva, respectively. Fitting  
 108 sequences to Greengenes and SILVA in closed reference mode performed similarly, with  
 109 median MCC scores of 0.8 and 0.72 respectively, while the median MCC dropped to  
 110 0.33 when fitting to RDP (see Figure 2). For comparison, clustering datasets *de novo*  
 111 with OptiClust produced an average MCC score of 0.83. This gap in OTU quality mostly  
 112 disappeared when clustering in open reference mode, which produced median MCCs of  
 113 0.82 with greengenes, 0.81 with SILVA, and 0.82 with RDP. Thus, open reference OptiFit  
 114 produced OTUs of very similar quality as *de novo* clustering, and closed reference OptiFit  
 115 followed closely behind as long as a suitable reference database was chosen.

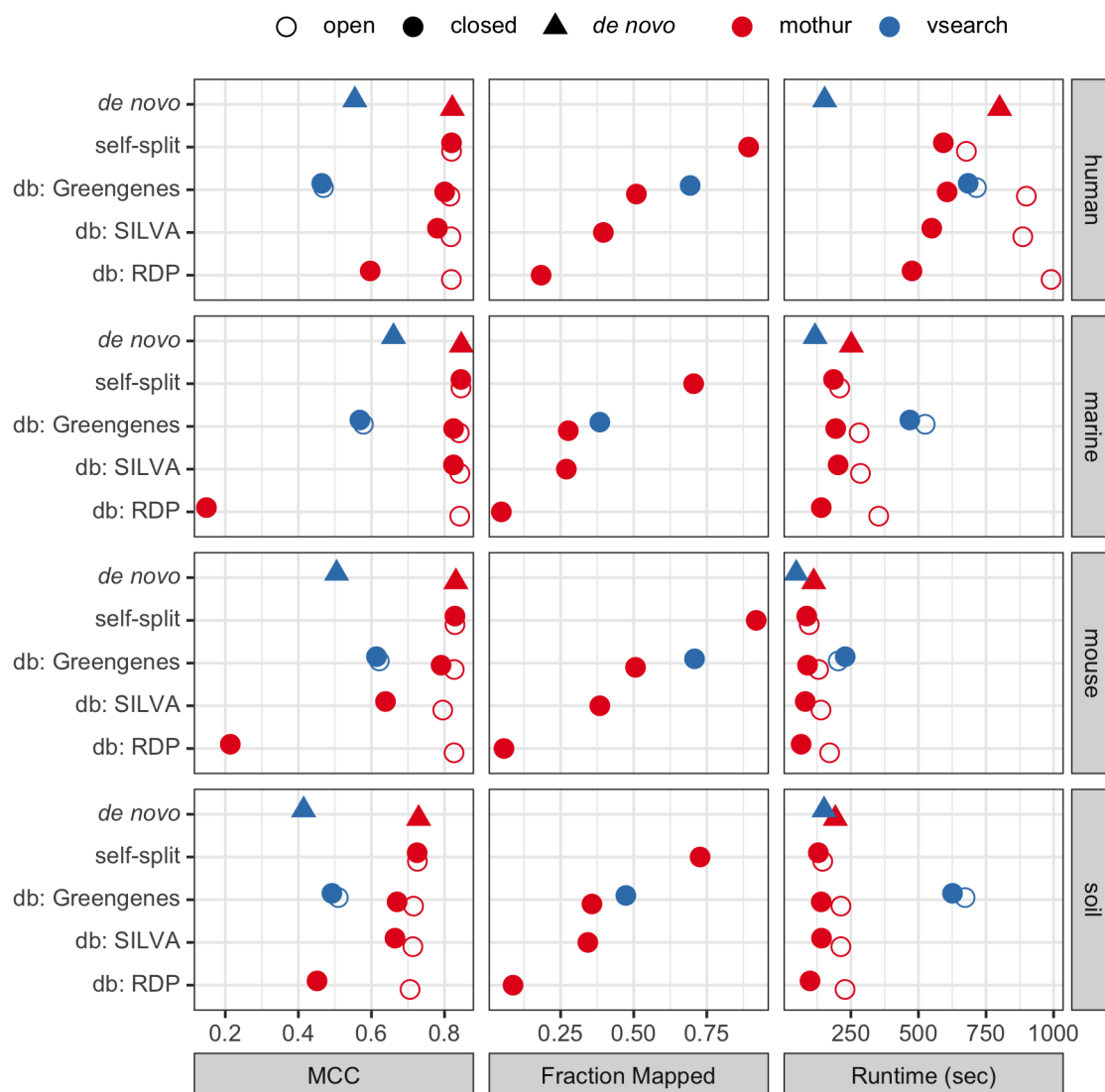


**Figure 1: The Analysis Workflow.** TODO write this caption.

Since closed reference clustering does not cluster query sequences that could not be assigned to reference OTUs, an additional measure of clustering performance to consider is the fraction of query sequences that were able to be assigned. On average, more sequences were assigned with Greengenes as the reference (43.1%) than with SILVA (36.4%) or RDP (7.1%). This mirrored the result reported above that Greengenes produced better OTUs in terms of MCC score than either SILVA or RDP. Note that *de novo* and open reference clustering methods always assign 100% of sequences to OTUs. The database chosen affects the final OTU assignments considerably in terms of both MCC score and fraction of query sequences that could be fit to the reference OTUs.

Despite the drawbacks, closed reference methods have been used when fast execution speed is required, such as when using very large datasets. To compare performance in terms of speed, we repeated each OptiFit and OptiClust run 100 times and measured the execution time. Across all dataset and database combinations, closed reference OptiFit outperformed both OptiClust and open reference OptiFit. For example, with the human dataset fit to SILVA reference OTUs, the average run times in seconds were 549.1 for closed reference OptiFit, 800.3 for *de novo* clustering the dataset, and 886.0 for open reference OptiFit. Thus, the OptiFit algorithm continues the precedent that closed reference clustering sacrifices OTU quality for execution speed.

To compare to the reference clustering method used by QIIME2, we clustered each dataset with VSEARCH against the Greengenes database of OTUs previously clustered at 97% sequence similarity. Each reference OTU from the Greengenes 97% database contains one reference sequence, and VSEARCH maps sequences to the reference based on each individual query sequence's similarity to the single reference sequence. In contrast, OptiFit accepts reference OTUs which each may contain multiple sequences, and the sequence similarity between all query and reference sequences is considered when assigning sequences to OTUs. *De novo* clustering with OptiClust produced 56.0%



**Figure 2: Benchmarking Results.** TODO write this caption.



higher quality OTUs than VSEARCH in terms of MCC, but performed 39.6% slower than VSEARCH. In closed reference mode, OptiFit produced 25.9% higher quality OTUs than VSEARCH, but VSEARCH was able to map 35.1% more query sequences than OptiFit to the Greengenes reference database. This is because VSEARCH only considers the distances between each query sequence to the single reference sequence, while OptiFit considers the distances between all pairs of sequences in an OTU. When open reference clustering, OptiFit produced higher quality OTUs than VSEARCH against the Greengenes database, with median MCC scores of 0.82 and 0.54 (respectively). In terms of run time, OptiFit outperformed VSEARCH in both closed and open reference mode by 74.3% and 135.3% on average respectively. Thus, the more stringent OTU definition employed by OptiFit resulted in fewer sequences being fit to reference OTUs than when using VSEARCH, but caused OptiFit to outperform VSEARCH in terms of both OTU quality and execution time.

## **Reference clustering with split datasets**

When performing reference clustering against public databases, the database chosen greatly affects the quality of OTUs produced. OTU quality may be poor when the reference database is too unrelated to the samples of interest, such as when samples contain low abundant or novel populations. While *de novo* clustering overcomes the quality limitations of reference clustering to databases, OTU assignments are not consistent when new sequences are added. Researchers may wish to fit new sequences to existing OTUs when comparing OTUs across studies or when making predictions with machine learning models. To determine how well OptiFit performs for fitting new sequences to existing OTUs, we employed a split dataset strategy, where each dataset was randomly split into a reference fraction and a query fraction. Reference sequences were clustered *de novo* with OptiClust, then query sequences were fit to the *de novo* OTUs with OptiFit.

167 First, we tested whether OptiFit performed as well as *de novo* clustering when using the  
168 split dataset strategy with half of the sequences selected for the reference by a simple  
169 random sample. OTU quality was highly similar to that from OptiClust regardless of mode  
170 (0.29% difference in median MCC). In closed reference mode, OptiFit was able to fit 81%  
171 of query sequences to reference OTUs with the split strategy, a great improvement over  
172 the average 43.1% of sequences fit to the greengenes database. In terms of run time,  
173 closed and open reference OptiFit performed faster than OptiClust on whole datasets by  
174 29.0% and 20.2 respectively. The split dataset strategy also performed 11.9% faster than  
175 the database strategy in closed reference mode and 30.4% faster in open reference mode.  
176 Thus, reference clustering with the split dataset strategy creates as high quality OTUs as  
177 *de novo* clustering yet at a faster run time, and fits far more query sequences than the  
178 database strategy.

179 While we initially tested this strategy using an even split of the data into reference and  
180 query fractions, we then wanted to investigate whether there was an optimal reference  
181 fraction size. To test the best reference size, reference sizes from 10% to 80% of the  
182 sequences were created, with the remaining sequences used for the query. OTU quality  
183 was remarkably stable across reference fraction sizes. For example, splitting the human  
184 dataset 100 times yielded a coefficient of variation of 0.00063 for the MCC score across all  
185 fractions. Run time generally decreased as the reference fraction increased; for the human  
186 dataset, the median run time was 762.8 with 10% of sequences in the reference and  
187 470.9 with 90% of sequences in the reference. In closed reference mode, the fraction of  
188 sequences that mapped increased as the reference size increased; for the human dataset,  
189 the median fraction mapped was 0.88 with 10% of sequences in the reference and 0.96  
190 with 90% of sequences in the reference. These trends hold for the other datasets as well  
191 (see supplemental figure **TODO**). Thus, the reference fraction does not affect OTU quality  
192 in terms of MCC score, but does affect the run time and the fraction of sequences that  
193 map during closed reference mode.

After testing the split strategy using a simple random sample to select the reference sequences, we then investigated other methods of splitting the data. We tested three methods for selecting the fraction of sequences to be used as the reference at a size of 50%; a simple random sample, weighting sequences by relative abundance, and weighting by similarity to other sequences in the dataset. OTU quality in terms of MCC was similar with the simple and abundance-weighted sampling (median MCCs of 0.82 and 0.84 respectively), but worse for similarity-weighted sampling (median MCC of 0.71). In closed-reference clustering mode, the fraction of sequences that mapped were similar for simple and abundance-weighted sampling (median fraction mapped of 0.96 and 0.95 respectively), but worse for similarity-weighted sampling (median fraction mapped of 0.85). While simple and abundance-weighted sampling produced better quality OTUs than similarity-weighted sampling, OptiFit performed faster on similarity-weighted samples with a median runtime of 113.1 seconds compared to 165.7 and 165.4 seconds for simple and abundance-weighted sampling (respectively). Thus, employing more complicated sampling strategies such as abundance-weighted and similarity-weighted sampling did not confer any advantages over selecting the reference via a simple random sample, and in fact decreased OTU quality in the case of similarity-weighted sampling.

## Discussion

We developed a new algorithm for fitting sequences to existing OTUs and have demonstrated its suitability for reference-based clustering. OptiFit makes the iterative method employed by OptiClust available for tasks where reference-based clustering is required. We have shown that OTU quality is similar between OptiClust and OptiFit in open reference mode, regardless of strategy employed. Open reference OptiFit performs slower than OptiClust due to the additional *de novo* clustering step, so users may prefer OptiClust for tasks that do not require reference OTUs.

When fitting to public databases, OTU quality dropped in closed reference mode to different degrees depending on the database and dataset source, and no more than half of query sequences were able to be fit to OTUs across any dataset/database combination. This may reflect limitations of reference databases, which are unlikely to contain sequences from rare and novel microbes. This drop in quality was most notable with RDP, which contains only about 21,000 sequences compared to over 200,000 sequences in SILVA and Greengenes each. Note that Greengenes has not been updated since 2013 at the time of this writing, while SILVA is updated every one to two years with the most recent release in 2019. We recommend that users who require an independent reference database opt for large databases with regular updates and good coverage of microbial diversity. Since OptiClust performs faster than open reference OptiFit and creates higher quality OTUs than closed reference OptiFit with the database strategy, we recommend using OptiClust rather than fitting to a database whenever stable OTUs are not required for the study at hand.

The OptiClust and OptiFit algorithms provided by mothur produced higher quality OTUs than VSEARCH in open reference, closed reference, or *de novo* modes. However, VSEARCH was able to map more sequences to OTUs than OptiFit in closed reference mode. While both mothur and VSEARCH use a distance or similarity threshold for determining how to assign sequences to OTUs, VSEARCH is more permissive than mothur. The OptiFit and OptiClust algorithms use all of the sequences to define an OTU, requiring that all pairs of sequences (including reference and query sequences) in an OTU are within the distance threshold without penalizing the MCC. In contrast, VSEARCH only requires each query sequence to be similar to the single sequence that seeded the OTU. In this way, VSEARCH sacrifices OTU quality in order to allow more sequences to fit to OTUs. Users who require closed reference clustering to the Greengenes database may prefer to use VSEARCH if they wish to maximize the fraction of sequences that can be fit at the cost of OTU quality. However, mothur's OptiClust or OptiFit are recommended for *de*

246 *de novo* or open reference clustering to produce OTUs of the highest possible quality.

247 When fitting with the split dataset strategy, OTU quality was remarkably similar when  
248 reference sequences were selected by a simple random sample or weighted by abundance,  
249 but quality was slightly worse when sequences were weighted by similarity. We recommend  
250 using a simple random sample since the more sophisticated reference selection methods  
251 do not offer any benefit. The similarity in OTU quality between OptiClust and OptiFit with  
252 this strategy demonstrates the suitability of using OptiFit to fit sequences to existing OTUs,  
253 such as when comparing OTUs across studies. However, when stable OTUs are not  
254 required, we recommend using OptiClust for *de novo* clustering over the split strategy with  
255 OptiFit since OptiClust is simpler to execute but performs similarly in terms of both run  
256 time and OTU quality.

257 We have developed a new clustering algorithm that allows users to produce high quality  
258 OTUs using already existing OTUs as a reference. Unlike existing reference-based  
259 methods which map query sequences to a single centroid sequence in each reference  
260 OTU, OptiFit considers all sequences in each reference OTU when fitting query sequences,  
261 resulting in OTUs of a similar high quality as those produced by the *de novo* OptiClust  
262 algorithm. Potential applications include fitting sequences to reference databases,  
263 comparing taxonomic composition of microbiomes across different studies, or using  
264 OTU-based machine learning models to make predictions on new data. OptiFit fills the  
265 missing option for fitting query sequences to existing OTUs that does not sacrifice OTU  
266 quality for stability of OTU assignments.

## Materials and Methods

### Data Processing Steps

We downloaded 16S rRNA gene amplicon sequences from four published datasets isolated from soil (7), marine (8), mouse gut (9), and human gut (10) samples. Raw sequences were processed using mothur according to the Schloss Lab MiSeq SOP as described in the mothur wiki and accompanying study by Kozich *et al.* (13, 14). These steps included trimming and filtering for quality, aligning to the SILVA reference alignment (11), discarding sequences that aligned outside the V4 region, removing chimeric reads with UCHIME (15), and calculating distances between all pairs of sequences within each dataset prior to clustering.

### Reference database clustering

To generate reference OTUs from independent databases, we downloaded sequences from the Greengenes database (v13\_8\_99) (6), SILVA non-redundant database (v132) (11), and the Ribosomal Database Project (v16) (12). These sequences were processed using the same steps outlined above followed by clustering sequences into *de novo* OTUs with OptiClust. Processed reads from each of the four datasets were clustered with OptiFit to the reference OTUs generated from each of the three databases. When reference clustering with VSEARCH, processed datasets were fit directly to the unprocessed Greengenes reference alignment, since this method is how VSEARCH is typically used by the QIIME2 software reference-based clustering (16, 17).

### Split dataset clustering

For each dataset, a fraction of the sequences was selected to be clustered *de novo* into reference OTUs with OptiClust. We used three methods for selecting the fraction of sequences to be used as the reference; a simple random sample, weighting sequences by

relative abundance, and weighting by similarity to other sequences in the dataset. Dataset splitting was repeated with reference fractions ranging from 10% to 80% of the dataset and for 100 random seeds. For each dataset split, the remaining sequences were assigned to the reference OTUs with OptiFit.

## Benchmarking

Since OptiClust and OptiFit employ a random number generator to break ties when OTU assignments are of equal quality, they produce slightly different OTU assignments when repeated with different random seeds. To capture any variation in OTU quality or execution time, clustering was repeated with 100 random seeds for each combination of parameters and input datasets. We used the benchmark feature provided by Snakemake to measure the run time of every clustering job. We calculated the MCC on each set of OTUs to quantify the quality of clustering, as described by Westcott *et al.* (1).

## Data and Code Availability

We implemented the analysis workflow in Snakemake (18) and wrote scripts in R (19), Python (20), and GNU bash (21). Software used includes mothur v1.45.0 (2), VSEARCH v2.13.3 (22), numpy (23), the Tidyverse metapackage (24), R Markdown (25), the SRA toolkit (26), and the conda environment manager (27). The complete workflow, manuscript, and conda environment are available at **TODO: UPDATED REPO LINK**.

## Acknowledgements

KLS received support from the NIH Training Program in Bioinformatics (T32 GM070449).

PDS received support from **TODO: Pat's grant(s)**.

The funders had no role in study design, data collection and interpretation, or the decision

to submit the work for publication.

## Author Contributions

KLS wrote the analysis code, evaluated the algorithm, and wrote the original draft of the manuscript. SLW designed and implemented the OptiFit algorithm and assisted in debugging the analysis code. MBM and GAD contributed analysis code. PDS conceived the study, supervised the project, and assisted in debugging the analysis code. All authors reviewed and edited the manuscript.

## References

1. **Westcott SL, Schloss PD.** 2017. OptiClust, an Improved Method for Assigning Amplicon-Based Sequence Data to Operational Taxonomic Units. *mSphere* **2**:e00073–17. doi:10.1128/mSphereDirect.00073-17.
2. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology* **75**:7537–7541. doi:10.1128/AEM.01541-09.
3. **Westcott SL, Schloss PD.** 2015. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ* **3**:e1487. doi:10.7717/peerj.1487.
4. **Topçuoğlu BD, Lesniak NA, Ruffin M, Wiens J, Schloss PD.** 2019. Effective application of machine learning to microbiome-based classification problems. *bioRxiv* 816090. doi:10.1101/816090.



5. **Schloss PD.** 2016. Application of a Database-Independent Approach To Assess the Quality of Operational Taxonomic Unit Picking Methods. *mSystems* **1**:e00027–16. doi:10.1128/mSystems.00027-16.
6. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL.** 2006. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *AEM* **72**:5069–5072. doi:10.1128/AEM.03006-05.
7. **Johnston ER, Rodriguez-R LM, Luo C, Yuan MM, Wu L, He Z, Schuur EAG, Luo Y, Tiedje JM, Zhou J, Konstantinidis KT.** 2016. Metagenomics Reveals Pervasive Bacterial Populations and Reduced Community Diversity across the Alaska Tundra Ecosystem. *Front Microbiol* **7**. doi:10.3389/fmicb.2016.00579.
8. **Henson MW, Pitre DM, Weckhorst JL, Lanclos VC, Webber AT, Thrash JC.** 2016. Artificial Seawater Media Facilitate Cultivating Members of the Microbial Majority from the Gulf of Mexico. *mSphere* **1**. doi:10.1128/mSphere.00028-16.
9. **Schloss PD, Schubert AM, Zackular JP, Iverson KD, Young VB, Petrosino JF.** 2012. Stabilization of the murine gut microbiome following weaning. *Gut Microbes* **3**:383–393. doi:10.4161/gmic.21008.
10. **Baxter NT, Ruffin MT, Rogers MAM, Schloss PD.** 2016. Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. *Genome Med* **8**:37. doi:10.1186/s13073-016-0290-3.
11. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO.** 2013. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research* **41**:D590–D596. doi:10.1093/nar/gks1219.

- 343 12. **Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT,**  
**Porras-Alfaro A, Kuske CR, Tiedje JM.** 2014. Ribosomal Database Project: Data  
and tools for high throughput rRNA analysis. *Nucl Acids Res* **42**:D633–D642.  
344 doi:10.1093/nar/gkt1244.
- 345 13. **Schloss PD, Westcott SL.** MiSeq SOP. [https://mothur.org/MiSeq\\_SOP](https://mothur.org/MiSeq_SOP).  
346
- 347 14. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013.  
Development of a Dual-Index Sequencing Strategy and Curation Pipeline for  
Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform.  
348 *Appl Environ Microbiol* **79**:5112–5120. doi:10.1128/AEM.01043-13.
- 349 15. **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R.** 2011. UCHIME  
improves sensitivity and speed of chimera detection. *Bioinformatics* **27**:2194–2200.  
350 doi:10.1093/bioinformatics/btr381.

- 351 16. **Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciulek T, Kreps J, Langille MGI, Lee J, Ley R, Liu Y-X, Lottfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Priesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG.** 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**:852–857. doi:10.1038/s41587-019-0209-9.
- 352
- 353 17. Clustering sequences into OTUs using Q2-vsearch — QIIME 2 2021.2.0  
354 documentation. <https://docs.qiime2.org/2021.2/tutorials/otu-clustering/>.
- 355 18. **Köster J, Rahmann S.** 2012. Snakemake — a scalable bioinformatics workflow  
356 engine. *Bioinformatics* **28**:2520–2522. doi:10.1093/bioinformatics/bts480.
- 357 19. **R Core Team.** 2020. R: A language and environment for statistical computing.  
358 Manual, R Foundation for Statistical Computing, Vienna, Austria.

20. **Van Rossum G, Drake FL.** 2009. Python 3 Reference Manual | Guide books.
21. Bash Reference Manual. <https://www.gnu.org/software/bash/manual/bash.html>.
22. **Rognes T, Flouri T, Nichols B, Quince C, Mahé F.** 2016. VSEARCH: A versatile open source tool for metagenomics. *PeerJ* **4**:e2584. doi:10.7717/peerj.2584.
23. **Harris CR, Millman KJ, van der Walt SJ, Gommers R, Virtanen P, Cournapeau D, Wieser E, Taylor J, Berg S, Smith NJ, Kern R, Picus M, Hoyer S, van Kerkwijk MH, Brett M, Haldane A, del Río JF, Wiebe M, Peterson P, Gérard-Marchant P, Sheppard K, Reddy T, Weckesser W, Abbasi H, Gohlke C, Oliphant TE.** 2020. Array programming with NumPy. *Nature* **585**:357–362. doi:10.1038/s41586-020-2649-2.
24. **Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H.** 2019. Welcome to the Tidyverse. *Journal of Open Source Software* **4**:1686. doi:10.21105/joss.01686.
25. **Xie Y, Allaire JJ, Grolemund G.** 2018. R Markdown: The Definitive Guide. Taylor & Francis, CRC Press.
26. SRA-Tools - NCBI. <http://ncbi.github.io/sra-tools/>.
27. 2016. Anaconda Software Distribution. Anaconda Documentation. Anaconda Inc.