

OVERVIEW

This document provides details, recommendations, and requirements for the amplicon library preparation services offered by the MSU Genomics Core. The table below provides an overview of the submission requirements for 16S-V4, 16S-V3V4, and all other amplicons. Additional requirements, PCR recipes, and cycling conditions are provided below the table.

The RTSF Genomics Core will include a negative, no template control (NTC) reaction on each plate of samples processed for amplicon sequencing, both 16S and other amplicons. Researchers must leave at least one well empty (H:12) on each plate submitted for amplicon library preparation. The NTC added by the Genomics Core substitutes 1 µl of nuclease-free water for the usual 1µl of template DNA in the PCR reaction. This reaction is processed and sequenced alongside your samples. The purpose for including this negative control is to demonstrate that good laboratory practices are being followed in the core facility, that no contamination of reagents or cross contamination of samples has occurred. Researchers are encouraged to include their own negative controls to monitor contamination introduced by sampling, DNA extraction and primary PCR reactions prior to sample submission to the Genomics Core.

For 16S-V4 and 16S-V3V4 amplicon projects the Genomics Core offers the option of including a positive control in your project, the ZymoBIOMICS Microbial Community DNA Standard (Zymo Research Cat# D6305/D6306). This mock community is composed of defined proportions of genomic DNA from eight bacteria plus two fungi, and sequencing of this mock community can serve as a useful positive control. Addition of a single positive control sample will be at no additional charge.

Region of Interest	Input	Concentration	Minimum Volume	Multiplex	Notes
16S-V4	Metagenomic DNA	1 ng/µl minimum	10 µl	up to 576 samples (dual-indexes)	Test amplification and gel image required, see below for more details.
16S-V3V4	Metaenomic DNA	1 ng/µl minimum	10 µl	up to 96 samples (single indexes)	Test amplification and gel image required, see below for more details.
Other	Primary PCR product	1 ng/µl minimum	10 µl	up to 576 samples (dual-indexes)	Gel image required, see below for more details.

16S-V4 AMPLICONS

The library preparation for the 16S-V4 region employs a one-step PCR method using the primer pair 515f/806r:

16S V4 forward (515f): GTGCCAGCMGCCGCGGTAA

16S V4 reverse (806r): GGACTACHVGGGTWTCTAAT

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and environmental microbiology 79, 5112–5120 (2013)

(https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP.md)

The submitter is required to:

- Quantify samples via fluorometric method (such as Qubit or PicoGreen) and normalize all samples to approximately the same concentration.
- Perform a test amplification using the 16S-V4 primers (above) to confirm the sample can be amplified and that the product is the expected size. A ladder must be run alongside the samples so that the PCR products can be sized. The gel must be run long enough to have good separation of the ladder. The gel image(s) must be provided at the time of submission.
- Submit samples in a 96-well PCR-type plate, filled by column, starting with A:1. Well H:12 must be left empty for the RTSF no template negative control.

The Genomics Core will use 1 µl of genomic DNA for the amplicon library preparation. Therefore, it is recommended that test amplifications be performed using a protocol similar to the Genomics Core's amplicon library preparation protocol:

<u>Component:</u>	<u>1 Rxn</u>
2X Dream Taq Master mix (K9012)	7.5 µl
0.5 µM Primer mix	6.5 µl
<u>DNA</u>	<u>1.0 µl</u>
Total	15 µl

16S-V4 Cycling conditions:

95°C for 3 minutes

30 cycles of:

95°C for 45 seconds

50°C for 60 seconds

72°C for 90 seconds

72°C for 10 minutes

15°C hold

16S-V3V4 AMPLICONS

The library preparation for the 16S-V3V4 region employs a one-step PCR method using the primer pair 341f/806r:

16S V3 forward (341f): CCTACGGGAGGCAGCAG

16S V4 reverse (806r): GGACTACHVGGGTWTCTAAT

Caporaso, J. G. et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences* 108 Suppl 1, 4516–4522 (2011)*

*The Genomics Core uses a modification of this protocol, substituting the 341f primer in place of the 515f primer.

The submitter is required to:

- Quantify samples via fluorometric method (such as Qubit or PicoGreen) and normalize all samples to approximately the same concentration.
- Perform a test amplification using the 16S-V3V4 primers (above) to confirm the sample can be amplified and that the product is the expected size. A ladder must be run alongside the samples so that the PCR products can be sized. The gel must be run long enough to have good separation of the ladder. The gel image(s) must be provided at the time of submission.
- Submit samples in a 96-well PCR-type plate, filled by column, starting with A:1. Well H:12 must be left empty for the RTSF no template negative control.

The Genomics Core will use 1 µl of genomic DNA for the amplicon library preparation. Therefore, it is recommended that test amplifications be performed using a protocol similar to the Genomics Core's amplicon library preparation protocol:

<u>Component:</u>	<u>1 Rxn</u>
2X Dream Taq Master mix (K9012)	7.5 µl
0.5 µM Primer mix	6.5 µl
<u>DNA</u>	<u>1.0 µl</u>
Total	15 µl

16S-V3V4 Cycling conditions:

95°C for 2 minutes

30 cycles of:

95°C for 40 seconds

50°C for 30 seconds

72°C for 60 seconds

72°C for 7 minutes

15°C hold

OTHER AMPLICONS (INDEX ADAPTERS ADDED BY MSU GENOMICS CORE)

For all other amplicon sequencing, a two-step PCR approach is used. The first PCR uses target-specific primers with tags on the 5' ends that allow the Genomics Core to do a second PCR for barcoding. The submitter is required to perform the primary PCR reaction. To do this, the submitter will need to order primers with the following tags on the 5' ends, with the target-specific forward and reverse sequences inserted in [TS-For] and [TS-Rev], respectively:

CS1-TS-F: 5' – ACACTGACGACATGGTTCTACA – [TS-For] – 3'

CS2-TS-R: 5' – TACGGTAGCAGAGACTTGGTCT – [TS-Rev] – 3'

When choosing primers for a targeted amplicon it is recommended that the product amplified is less than 400bp (not including the 44bp added by the CS1/CS2 overhangs). This size will permit adequate overlap of the forward and reverse read 3' ends when sequencing with a 2x250bp format.

The Genomics Core will use 1 µl of primary PCR product for the amplicon indexing.

The submitter is required to:

- Quantify samples via fluorometric method (such as Qubit or PicoGreen) and normalize all samples to approximately the same concentration.
- Run products on a gel to confirm the amplification was successful, the products are the expected size, and that off-target products (including primer-dimer) are not present. The gel image must be provided at the time of submission.
- Products do not need to be cleaned up prior to submission. However, if primer dimers or off-target products are generated, then products should be cleaned up to remove these products and/or the primary PCR reaction should be optimized.
- Submit samples in a 96-well PCR-type plate, filled by column, starting with A:1. Well H:12 must be left empty for the RTSF no template negative control.