



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L704

Amplicon Library Preparation for Sequencing

Standard Operating Procedure

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1. Purpose

This SOP describes the preparation of amplicon sequencing libraries for Illumina platforms, following a two-step PCR approach. The first PCR amplifies the target marker gene (e.g., 16S rRNA for bacteria, ITS for fungi) with gene-specific primers tailed with Illumina adapter overhangs. The second PCR attaches unique dual-index barcodes and the full Illumina adapter sequences, enabling multiplexed sequencing of many samples per run. This protocol is based on the Illumina 16S Metagenomic Sequencing Library Preparation guide and the Earth Microbiome Project (EMP) standards (Caporaso et al., 2012; Illumina, Inc., 2013; Thompson et al., 2017).

2. Scope

- **Applies to:** Preparation of amplicon libraries targeting 16S rRNA (V4 or V3–V4 region), ITS (ITS1 or ITS2 region), 18S rRNA, or other marker genes from environmental DNA extracts for paired-end sequencing on Illumina MiSeq or NextSeq platforms
- **Does not apply to:** Shotgun metagenomic library preparation; long-read amplicon sequencing (PacBio, Nanopore); ddPCR (see [SOP-L701](#)); eDNA metabarcoding using non-Illumina workflows
- **Location:** Molecular biology bench, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve target region, primer choice, and sequencing facility
Trained Personnel	Prepare libraries per this SOP; submit to sequencing facility
New Students	Complete training before independent use

4. Definitions

- **Adapter:** A short double-stranded DNA sequence ligated to both ends of a library fragment; contains the sequencing primer binding site, an index sequence, and the flow cell binding sequence required for Illumina cluster generation.
- **Amplicon library:** A sequencing library prepared by PCR amplification of a target region (e.g., 16S rRNA gene V4 region or ITS2) using primers that incorporate Illumina adapter sequences; represents the diversity of that locus in a community sample.
- **Index (barcode):** A unique 6–12 bp sequence incorporated into the adapter of each sample's library; allows multiple samples to be pooled and sequenced together (multiplexed) and computationally separated (demultiplexed) after sequencing.
- **Library normalization:** Adjustment of each sample's library to the same molar concentration before pooling; ensures approximately equal sequencing depth across samples; performed by quantification (Qubit or qPCR) followed by dilution.

- **Library pooling:** Combining equimolar amounts of individually indexed libraries from multiple samples into a single tube for multiplexed sequencing; the total library pool is loaded onto the sequencer.
- **Paired-end sequencing:** A sequencing strategy in which both ends of a DNA insert are sequenced, producing two reads (R1 and R2) per cluster; paired reads overlap for amplicons shorter than twice the read length, enabling error correction by merging.
- **PCR inhibitor:** A substance carried over from DNA extraction that suppresses amplification efficiency (e.g., humic acids, tannins, residual ethanol); detected by inhibition testing and remedied by further purification or dilution of the template.
- **Size selection:** A step in library preparation that removes off-target fragments (primer dimers, non-specific products) using bead-based cleanup (e.g., AMPure XP beads) or gel excision; improves sequencing quality and reduces wasted reads.

5. Safety and Hazards

Hazards

- **Chemical:** AMPure XP beads (or equivalent SPRI beads) contain polyethylene glycol (PEG) and carboxylated magnetic particles; low toxicity but handle with gloves. Molecular-grade ethanol is flammable.
- **Biological:** Environmental DNA extracts may contain pathogen-derived DNA. Handle with standard precautions.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves (changed frequently)

5.2. Emergency Procedures

Refer to [SOP-L003 Hazardous Materials Handling](#).

6. Equipment and Materials

6.1. Equipment

Item	Location	Notes
Thermal cycler	Mol bio bench	For both PCR1 and PCR2
Magnetic stand (for 96-well plates or strip tubes)	Mol bio bench	For SPRI bead cleanup
Qubit 4 fluorometer	Instrument bench	For library quantification

Item	Location	Notes
NanoDrop One	Instrument bench	Optional purity check
Gel electrophoresis system	Mol bio bench	For amplicon verification; see SOP-L703
Multichannel pipette (optional)	Mol bio bench	For high-throughput plate work
Plate vortex mixer	Mol bio bench	For mixing bead suspensions
Microcentrifuge and plate spinner	Mol bio bench	For brief spin-downs

6.2. Reagents and Consumables

Item	Notes
High-fidelity DNA polymerase	e.g., KAPA HiFi HotStart (Roche), Q5 (NEB), or Phusion (Thermo Fisher); high-fidelity polymerase is required to minimize PCR-introduced errors (Schirmer et al., 2015)
Gene-specific primers with Illumina overhangs (PCR1)	See Section 6.1
Nextera XT Index Kit v2 (or equivalent dual-index primers)	For PCR2 barcoding
AMPure XP beads (Beckman Coulter) or equivalent SPRI beads	For size selection and cleanup
Molecular-grade ethanol (80%, freshly prepared)	For bead washes
Nuclease-free water	For elution and dilutions
Qubit dsDNA HS Assay Kit	For library quantification
Agarose and gel supplies	For amplicon verification
PCR plates or strip tubes (0.2 mL)	Low-bind preferred
Plate sealing film or strip caps	
Filter pipette tips	Barrier tips mandatory

7. Procedure

7.1. Primer Design (PCR1: Gene-Specific Amplification)

Primers for the first PCR consist of the gene-specific sequence plus the Illumina adapter overhang. The overhang sequences are:

- Forward overhang: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[gene-specific forward]-3'
- Reverse overhang: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[gene-specific reverse]-3'

Commonly used gene-specific primers:

Target	Primers	Region	Amplicon	Reference
16S bacteria/archaea	515F / 806R	V4	~290 bp	Caporaso et al. (2012); Parada et al. (2016)
16S bacteria	341F / 785R	V3–V4	~460 bp	Klindworth et al. (2013)
ITS fungi	ITS1f / ITS2	ITS1	~250–400 bp	Smith & Peay (2014); White et al. (1990)
ITS fungi	ITS7 / ITS4	ITS2	~250–400 bp	Ihrmark et al. (2012)
18S eukaryotes	Euk1391f / EukBr	V9	~130 bp	Amaral-Zettler et al. (2009)

i Note

The EMP standard primers for 16S V4 are 515F (Parada) / 806R (Apprill), which include degeneracies for improved archaeal coverage (Apprill et al., 2015; Parada et al., 2016). Use these unless the project specifically requires a different region.

7.2. PCR1: Target Amplification

1. Prepare the master mix on ice. Use a high-fidelity polymerase.

Component	Volume per reaction	Final concentration
2x KAPA HiFi HotStart ReadyMix	12.5 uL	1x
Forward primer with overhang (10 uM)	1.0 uL	400 nM
Reverse primer with overhang (10 uM)	1.0 uL	400 nM
Nuclease-free water	8.5 uL	–
Template DNA (1–10 ng)	2.0 uL	Variable
Total	25.0 uL	

2. Include controls: positive control, NTC, and extraction blank.
3. Cycling parameters (adjust annealing temperature per primer set):

Step	Temperature	Time	Cycles
Initial denaturation	95 C	3 min	1
Denaturation	95 C	30 s	25–28
Annealing	55 C	30 s	25–28
Extension	72 C	30 s	25–28
Final extension	72 C	5 min	1
Hold	4 C	Hold	–

! Important

Limit PCR1 to 25–28 cycles to minimize chimera formation and PCR bias (Schirmer et al., 2015). More cycles increase the proportion of artifacts in the sequencing data.

4. Verify amplification by running 2 uL on a 1.5% agarose gel per [SOP-L703](#).

7.3. PCR1 Cleanup (SPRI Beads)

1. Allow AMPure XP beads to reach room temperature (approximately 30 minutes). Vortex well before use.
2. Add 20 uL (0.8x ratio) of beads to each 25 uL PCR product. Mix by pipetting 10 times.
3. Incubate at room temperature for 5 minutes.
4. Place on the magnetic stand for 2 minutes until the solution clears.
5. Aspirate and discard the supernatant.
6. Wash twice with 200 uL of freshly prepared 80% ethanol. Do not disturb the bead pellet. Allow 30 seconds per wash.
7. Remove all ethanol and air-dry the beads for 3–5 minutes. Do not over-dry (beads will crack and yield drops).
8. Remove from the magnetic stand. Elute in 27.5 uL of nuclease-free water. Pipette to mix.
9. Return to the magnetic stand. Transfer 25 uL of the clear eluate to a new tube.

7.4. PCR2: Index PCR (Barcoding)

1. Assign a unique dual-index combination (i7 + i5) to each sample. Record the plate map in the lab notebook and in the sample sheet.
2. Set up the index PCR:

Component	Volume per reaction
2x KAPA HiFi HotStart ReadyMix	12.5 uL
Nextera XT Index Primer 1 (i7)	2.5 uL
Nextera XT Index Primer 2 (i5)	2.5 uL
Cleaned PCR1 product	2.5 uL
Nuclease-free water	5.0 uL
Total	25.0 uL

3. Cycling parameters:

Step	Temperature	Time	Cycles
Initial denaturation	95 C	3 min	1
Denaturation	95 C	30 s	8
Annealing	55 C	30 s	8

Step	Temperature	Time	Cycles
Extension	72 C	30 s	8
Final extension	72 C	5 min	1
Hold	4 C	Hold	–

4. Clean the indexed PCR product with AMPure XP beads (0.8x ratio) as in Section 6.3. Elute in 27.5 uL.

7.5. Library Quantification and QC

1. Quantify each library using the Qubit dsDNA HS Assay ([SOP-L700](#), Section 6.4).
2. Verify library size by running 1 uL on a 1.5% agarose gel. The expected size is the amplicon plus approximately 120 bp of adapter and index sequence.
3. Libraries with concentrations below 1 nM may not sequence well. Re-amplify or exclude them.

7.6. Library Pooling and Submission

1. Normalize each library to an equimolar concentration (typically 4 nM) by diluting with nuclease-free water.
2. Pool equal volumes of all normalized libraries into a single tube.
3. Quantify the pooled library by Qubit.
4. Prepare the sample sheet (Illumina Experiment Manager format or the sequencing facility's template) listing every sample with its index combination.
5. Submit the pooled library and sample sheet to the sequencing facility per their submission guidelines.
6. Record the submission date, facility, run type (e.g., MiSeq 2x250 or 2x300), and expected turnaround in the lab notebook.

8. Quality Control

- Every library prep run must include: positive control (mock community, if available), NTC, and extraction blank carried through both PCR1 and PCR2
- NTC and extraction blanks must show no visible band on the verification gel and no detectable concentration on the Qubit. If blanks are positive, the entire batch is suspect (Salter et al., 2014).
- Verify amplicon size on gel after PCR1; verify library size (amplicon – adapters) on gel after PCR2
- Record the number of PCR1 cycles and the index assignments for every sample; these are required for data analysis and troubleshooting

9. Waste Disposal

Waste Type	Container	Disposal Method
Used SPRI beads	Standard trash	Non-hazardous
Ethanol wash waste	Chemical waste or evaporate in fume hood	Small volumes can evaporate
Used PCR tubes, plates, tips	Standard trash	Non-hazardous
Gel waste	Per SOP-L703	Depends on stain used

10. References

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11. Related SOPs

SOP	Title	Relationship
L700	DNA Extraction	Upstream: provides template DNA
L703	PCR and Gel Electrophoresis	Amplicon verification
L705	Bioinformatics Pipeline	Downstream: sequence processing
L706	eDNA Metabarcoding	eDNA-specific workflow
F701	Soil Microbial Sampling	Field collection

12. Training Record

Before preparing amplicon libraries independently, personnel must:

1. Read this SOP, [SOP-L703](#), and [SOP-L700](#) in full
2. Demonstrate SPRI bead cleanup with correct bead ratios and wash steps
3. Prepare a supervised library from positive control DNA through pooling, with clean controls and correct library size on gel
4. Complete the sequencing facility's sample submission training
5. Be signed off by the PI or lab manager