Microsynth

Amplicon Sequencing Sample Preparation (1st Step PCR Products)

This document provides essential guidance for generating high-quality PCR products (1st Step PCR) for submission to Microsynth. Since 1st Step PCR is performed outside of our control, following these recommendations is critical to ensure optimal sequencing results.

Overview & Applications

Amplicon sequencing is commonly used for:

- CRISPR screening and validation (on/off-target analysis)
- Somatic variant analysis
- Taxonomic identification (16S, 18S, ITS, COI, rbcL, etc.)
- Antibody discovery (scFv, nanobodies, sdAb, etc.)
- Diversity analysis (e.g., synthetic DNA libraries, phage display libraries)

Amplicon Requirements

- Target amplicon size (excluding Illumina overhangs): **150–480 bp**. Optional heterogeneity spacers count towards the total amplicon length.
- Amplicons should be specific, clean, and single-band products
- Avoid smears or multiple bands

Primer Design

General Considerations

Primer design strongly impacts sequencing success.

- Use validated primers from literature when possible
- Ensure high specificity (avoid off-target amplification)
- Aim for uniform amplicon length distribution

Illumina-Specific Recommendations

- Design amplicons to achieve a minimum overlap of ~50 bp between paired-end reads for robust read merging.
- Avoid low sequence diversity at the start of sequencing reads, as this can reduce sequencing quality.
  - Heterogeneity spacers (N bases) may be added between adapter and primer to increase sequence diversity, particularly for low-diversity amplicon pools. Spacer bases count towards the total amplicon length.

Adapter Sequences (Required)

Use Illumina Nextera adapter overhangs in your primers:

- **Forward primer:**
  `5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]`

- **Reverse primer:**
  `5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]`

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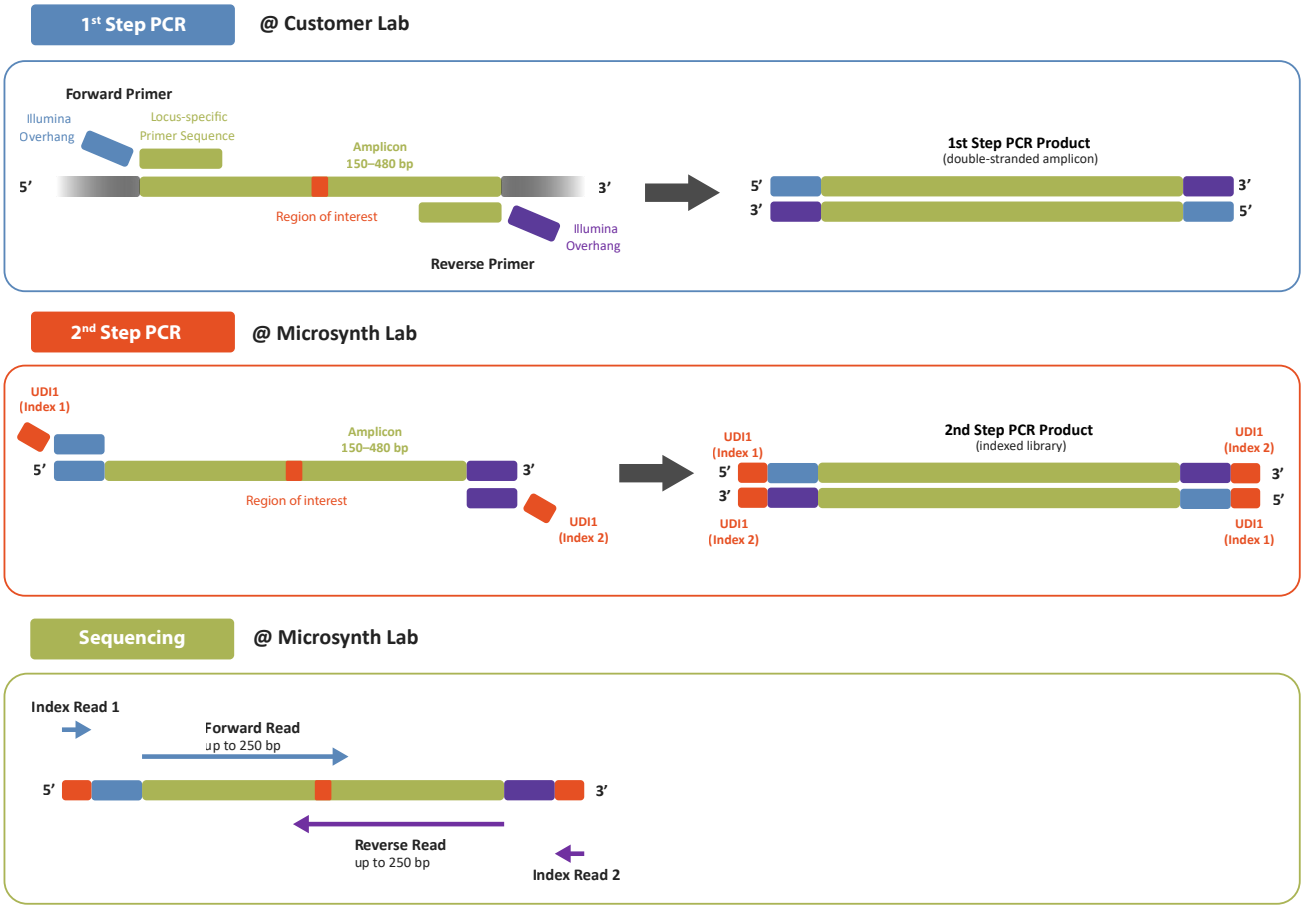

Key Point
You provide 1st step PCR products with Illumina Nextera adapter overhangs. We add the indexes and prepare the library for sequencing.

Note: *Optional heterogeneity spacers are not required but may improve sequencing performance for low-diversity amplicons.*

Primer Properties

- Locus-specific region Tm: **60–65°C**
- Standard desalting purification is sufficient for most applications.
- Use high-quality oligonucleotides for both forward and reverse primers (e.g., Microsynth primers).
- For challenging PCR assays or CRISPR on-target frequency analysis, IEX-HPLC purified primers are recommended.

1st Step PCR Setup & Optimization

Polymerase Selection

- Genome editing: Use proofreading polymerase
- Metabarcoding: Use robust polymerase for complex samples

Primer Concentration

- Typical: 0.2–0.5 µM (each primer)
- Avoid excessive concentrations (can lead to dimers and artifacts)

Annealing Temperature
- Determine experimentally (e.g., gradient PCR)
- Do not rely solely on calculated Tm

Cycle Number
- Recommended starting point: ~25 cycles
- Minimum: 20 cycles
- Avoid overcycling → reduces data quality and increases artifacts

Sample Input Considerations

- Ensure sufficient **target DNA representation**
- Consider sample complexity (e.g., host DNA background in metabarcoding)
- For genome editing, ensure enough edited copies for detection

Controls

We strongly recommend including:
- **Positive controls** (to confirm amplification success)
- **Negative controls** (to detect contamination)

Positive and negative controls are optional but recommended to verify your experimental workflow. If submitted, please clearly identify control samples.
Negative controls are processed and sequenced like regular samples and will therefore be charged accordingly. Proper labeling helps avoid delays in turnaround time when no reads are expected.

Quality Control Before Submission (Mandatory)

Purification
- Purify PCR products before performing final quality control and quantification.
- Recommended: bead-based purification (≥1.2× ratio)

Qualitative Analysis
- Confirm correct product size using:
  - Agarose gel OR
  - Capillary electrophoresis
- **Do not submit samples without visible PCR product**

Quantification
- Use fluorescence-based methods (e.g., Qubit, PicoGreen)
- Normalize samples to **1 ng/µL** using water, EB (elution buffer), or low-EDTA TE buffer (<0.1 mM EDTA). Submit at least **20 µL.**

Sample Submission & Shipment

- Ship at ambient or cooled temperature
- Avoid dry ice for plates with adhesive seals
- 96-well plates are preferred over individual tubes
- Fill 96-well plates column-wise without gaps between submitted samples.
- Ensure proper labeling and documentation

Common Issues and their Impact

Issue	Impact
No purification	Poor library quality, inaccurate quantification
Amplicons outside recommended size range	Reduced read quality or incomplete sequencing
Multiple PCR products	Uneven read distribution
No QC performed	No guarantee of sequencing success
Unexpected product size	Reduced data quality/quantity
Low sample concentration	Insufficient reads, sampling error
Samples not normalized to 1 ng/µL	Uneven read distribution between samples

Responsibilities

Not Under Microsynth Control

- 1st Step PCR amplification quality
- PCR product purification
- Sample normalization (equal volumes are processed)
- Chimera formation during PCR
- Coverage uniformity across amplicon
- Per-sample read distribution (depends on input balance)
- Qualitative and quantitative QC of submitted samples

Controlled by Microsynth

- Shipment integrity check upon arrival
- Index PCR and library preparation
- Removal of free adapters and primer dimers (following indexing step)
- Library quantification and equal mass pooling
- Sequencing quality metrics, including:
  - ≥60% Pass Filter (PF) reads
  - Low error rates (PhiX <2%)
  - High base quality metrics, with typical Q30 values exceeding 75–85% depending on run configuration

Key Takeaways

- **1st Step PCR quality is critical** for sequencing success
- Submit **clean, specific, and quantified amplicons**
- Perform both qualitative and quantitative QC **after purification**
- Normalize samples to **1ng/µL** before submission
- Follow primer and amplicon design recommendations carefully

Need More Information?

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