

Balancing cost, DNA input, and performance across HiFi microbial and metagenomic WGS workflows

Comparing standard HiFi library preparation, the Ampli-Fi protocol, and seqWell LongPlex™ multiplexing for microbial and metagenomic applications on the Vega™ system

Overview

High-quality long-read sequencing enables complete microbial genome assembly and high-resolution metagenomic analysis, but DNA input, workflow complexity, and per-sample reagent cost can constrain study design. This Technical note compares four implementations of three HiFi library preparation strategies using 16 bacterial isolates and two metagenomic reference materials. After downsampling to approximately match read counts, all workflows produced similar isolate and metagenome assembly outcomes; the principal differences were input requirement, mean read length, methylation retention, preparation time, demultiplexing behavior, and estimated reagent cost.

Key findings

Longest mean HiFi reads

Standard HiFi preparation produced the longest mean HiFi reads and retained methylation information.

Lowest DNA input

The Ampli-Fi protocol supported inputs as low as 1 ng and enabled barcoding and pooling before SMRTbell® library preparation.

Lowest estimated reagent cost and easiest workflow

seqWell LongPlex provided shearing and barcoding in one step and an estimated per-sample library reagent cost starting at approximately \$23.

Comparable assembly outcomes

Within the evaluated samples and downsampling design, all workflows delivered similar isolate and metagenome assembly results.

PacBio® HiFi sequencing combines long reads with high single-molecule accuracy, supporting microbial genome assembly, variant detection, metagenomic profiling, and metagenome assembly. As projects scale, laboratories increasingly need workflows that reduce input requirements, simplify preparation, and lower per-sample cost without compromising the assembly results required for microbial and metagenomic applications.

This evaluation compared standard HiFi library preparation, the Ampli-Fi protocol, and seqWell LongPlex multiplexing. LongPlex was evaluated in both PCR+ and PCR-free configurations. The comparison focused on workflow simplicity, DNA input, mean HiFi read length, read quality, yield, barcoded and unique reads, duplicate rates, assembly performance, preparation time, and estimated library reagent cost.

Study at a glance

Samples	16 bacterial isolates; ZymoBIOMICS Gut Microbiome Standard (D6331); ZymoBIOMICS Fecal Reference (D6323)
Workflows	Standard HiFi; Ampli-Fi; seqWell LongPlex PCR+; seqWell LongPlex PCR-free
Sequencing	PacBio Vega system; 24-hour runs
Assembly	hifiasm for isolates; hifiasm-meta and myloasm for metagenomes; HiFi-MAG-Pipeline for binning, QC, and MAG construction
Normalization	Data were downsampled to approximately match the sample with the fewest reads; isolate coverage analysis used 200 Mb, except one 170 Mb sample

Practical interpretation

These workflows are best viewed as complementary options. Standard HiFi prioritizes read length and methylation; Ampli-Fi prioritizes very low input; and LongPlex prioritizes workflow simplicity and estimated per-sample cost, with PCR+ and PCR-free versions providing different trade-offs.

Library preparation options

Workflow	DNA input	Manual prep time*	Estimated cost/sample**	Methylation	Primary strengths	Primary considerations
Standard HiFi	50-500 ng	3.5-6.5 h	~\$40+	Retained	Maximum mean HiFi read length; multiplexing up to 384.	Higher reagent cost and input requirement than the alternative workflows.
Ampli-Fi	As low as 1 ng	~7 h	~\$46+	Not retained	Lowest input; upfront barcoding and pooling; access to challenging sample types; CiFi application.	Slightly shorter mean reads and higher estimated reagent cost.
LongPlex PCR+	≥150 ng	~5.5 h for 96 samples	~\$25+	Not retained	Shearing and barcoding in one step; simple workflow; faster turnaround; lowest estimated cost.	Shorter mean reads; PCR amplification removes methylation information.
LongPlex PCR-free	≥150 ng	~5.5 h for 96 samples	~\$23+	Retained	Simple workflow; lowest estimated cost; maintained methylation; increased plasmid recovery in this study.	Lower demultiplexing rates for metagenomic, fragmented, or lower-molecular-weight DNA.

* Manual preparation time without SRE; optional QC checkpoints are not included. ** Prices are in USD, may vary by region, kit, and plexity, and include library reagents only. Instrument amortization, other reagents, sequencing, and DNA extraction are excluded.

Experimental design and analysis

Each workflow was applied to genomic DNA from 16 bacterial isolates and two metagenomic reference materials. All libraries were sequenced on the Vega system using 24-hour runs. The downstream processing path depended on whether the workflow introduced a second barcode layer or PCR duplicates.

Sequencing performance

Standard HiFi library prep produced the longest mean reads for all three sample groups: 6.32 kb for the gut microbiome standard, 7.74 kb for the pooled fecal reference, and 15.29 kb

across the 16 bacterial isolates. Median read quality across the full study ranged from Q35 to Q51. Final HiFi yield varied with sample type, workflow, and pooling design, from 2.01 Gb to 64.6 Gb in the values reported here.

Duplicate rates were low in the workflows that included deduplication, ranging from 0.007% to 1.06%. Upstream demultiplexing rates varied substantially by sample and workflow; however, interpreting these percentages as a direct measure of success is cautioned against because the run pooling strategies were not equivalent.

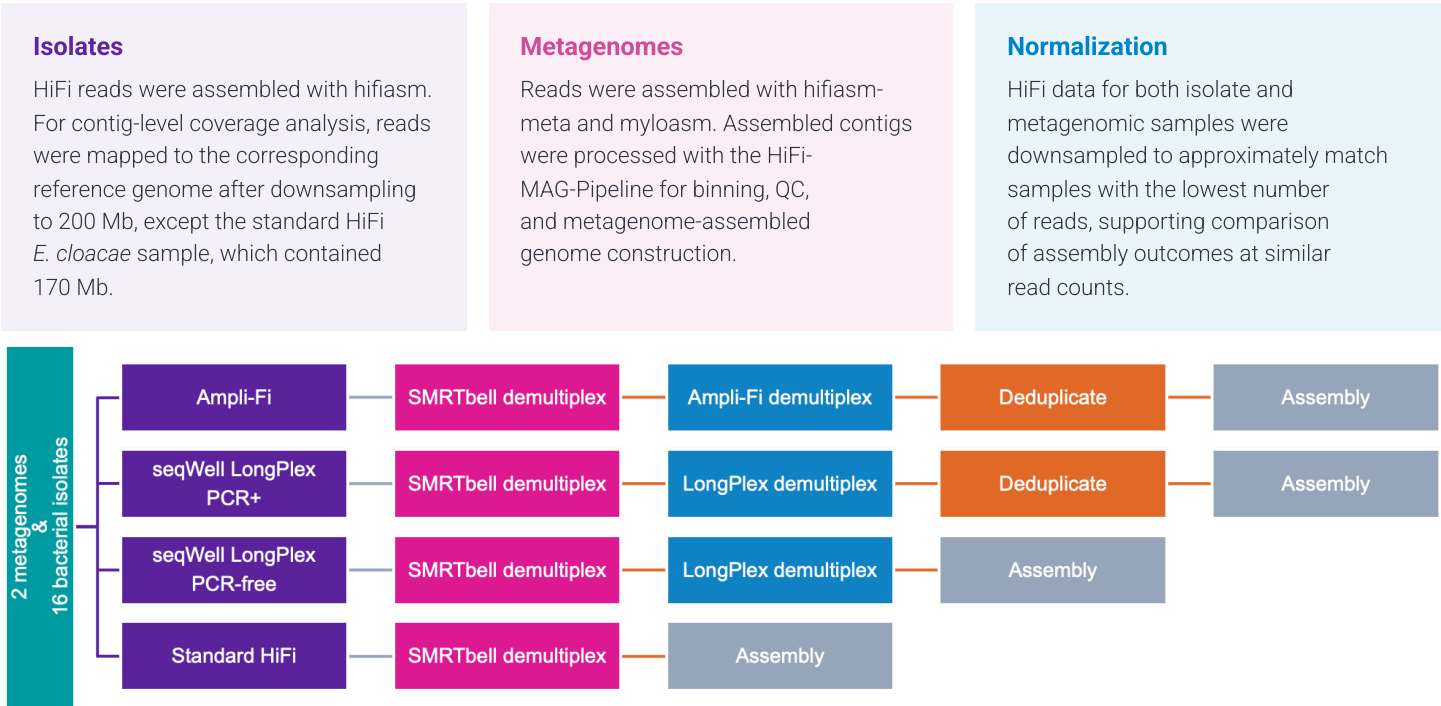


Figure 1. Experimental design and analysis workflow. Ampli-Fi and LongPlex PCR+ data underwent workflow-specific demultiplexing and deduplication before assembly. LongPlex PCR-free data underwent LongPlex demultiplexing without deduplication, and standard HiFi data proceeded from SMRTbell demultiplexing directly to assembly.

Sample group	Standard HiFi	Ampli-Fi	LongPlex PCR+	LongPlex PCR-free
Gut standard	6.32 kb Q51 2.52 Gb	5.76 kb Q50 2.01 Gb	4.84 kb Q48 9.80 Gb	5.15 kb Q50 3.52 Gb
Pooled fecal reference	7.74 kb Q47 32.4 Gb	6.55 kb Q48 20.9 Gb	5.38 kb Q46 49.8 Gb	5.63 kb Q49 13.8 Gb
16 bacterial isolates	15.29 kb Q35 64.6 Gb	7.98 kb Q42 57.4 Gb	5.93 kb Q44 62.3 Gb	7.84 kb Q46 21.8 Gb

Table 1. Values are mean HiFi read length | median read quality | final HiFi yield. Complete barcoding, demultiplexing, duplicate, and unique-read metrics are provided in Appendix A.

Isolate assembly performance

After downsampling the isolate data to 200 Mb, reads were mapped to the respective reference genomes to evaluate both mean contig coverage and the percentage of each contig that achieved at least 10x coverage. The standard HiFi *E. cloacae* sample contained 170 Mb and was not further downsampled.

The seqWell LongPlex preparations, both PCR+ and PCR-free, showed the fewest contig coverage dropouts. The remaining pronounced dropouts were concentrated in the smallest

contigs of *K. pneumoniae* and *E. cloacae*. All workflows missed the same regions of *E. cloacae* reference contigs 2 and 4, a pattern consistent with reference divergence rather than a workflow-specific loss of coverage. Across the full isolate panel, the assembly comparison supported the conclusion that all four library preparation paths produced similar overall assembly outcomes with seqWell LongPlex capturing more plasmid contigs.



Figure 2. Contig-level coverage of microbial isolates. Left: percentage of each reference contig with at least 10x coverage. Right: mean contig coverage. Data were downsampled to 200 Mb, except the standard HiFi *E. cloacae* sample, which contained 170 Mb.

Sample	Assembler	Standard library prep	Ampli-Fi	LongPlex PCR+	LongPlex PCR-free
Zymo gut standard	hifiasm-meta	14	11	11	12
Zymo gut standard	myloasm	13	11	11	12
Pooled fecal reference	hifiasm-meta	135	134	108	118
Pooled fecal reference	myloasm	159	137	128	137

Table 2. Total MAG counts shown in Figure 3. Totals combine sc-HQ, mc-HQ, and MQ MAG categories.

Metagenome-assembled genome performance

Metagenomic reads from the Zymo gut microbiome standard and the Zymo pooled human fecal reference were assembled with hifiasm-meta and myloasm, then processed through the HiFi-MAG-Pipeline. Data for each sample were downsampled to approximately match the lowest Gb before assembly comparison.

All evaluated library preparation methods delivered similar assembly results for bacterial isolates and metagenomic samples after downsampling to comparable read counts. These findings support selecting a HiFi library preparation method according to the specific input, cost, methylation, and workflow requirements of a project or laboratory.

Standard HiFi library preparation is the strongest fit when maximum mean read length and methylation information are priorities. Ampli-Fi extends HiFi sequencing to DNA inputs as low as 1 ng and supports barcoding before SMRTbell library preparation. seqWell LongPlex offers the lowest estimated per-sample reagent cost and the simplest preparation path through combined shearing and upfront barcoding, with PCR+ and PCR-free options providing different methylation and demultiplexing trade-offs.

Across the evaluated plexities and genome sizes, all workflows achieved estimated per-sample library reagent costs below \$50, enabling high-throughput applications including microbial isolate assembly, metagenome profiling, and metagenome assembly.

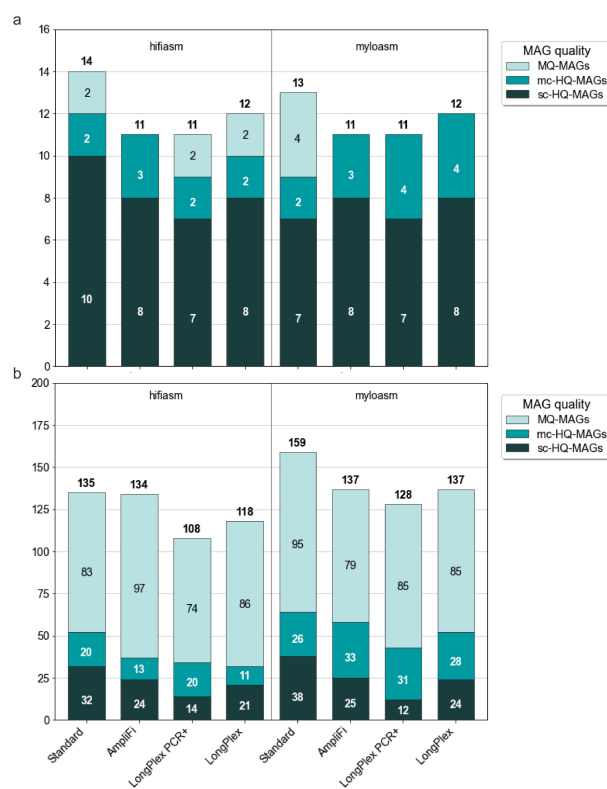


Figure 3. Metagenome-assembled genome results for (a) the Zymo gut microbiome standard and (b) the Zymo pooled human fecal reference. sc-HQ-MAGs are single-contig high-quality MAGs (>90% completeness and <5% contamination); mc-HQ-MAGs are multiple-contig high-quality MAGs; MQ-MAGs are medium-quality MAGs (>50% completeness and <10% contamination). The figure label "hifiasm" corresponds to the hifiasm-meta analysis described in the study workflow.

REFERENCES

1. PacBio HiFi library preparation kits
2. Ampli-Fi protocol: Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing
3. Cheng H, Concepcion GT, Feng X, Zhang H, Li H. (2021). Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. Nature Methods. 18(2):170-175. <https://doi.org/10.1038/s41592-020-01056-5>
4. Feng X, Cheng H, Portik D, Li H. (2022). Metagenome assembly of high-fidelity long reads with hifiasm-meta. Nature Methods. 19(6):671-674. <https://doi.org/10.1038/s41592-022-01478-3>
5. Shaw J, Marin MG, Li H. (2026). High-resolution metagenome assembly for modern long reads with myloasm. Nature Biotechnology. <https://doi.org/10.1038/s41587-026-03053-z>

RESOURCES

1. PacBio HiFi library preparation kits
2. Ampli-Fi protocol: Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing
3. [seqWell LongPlex multiplexing kit](#)
4. [ZymoBIOMICS Gut Microbiome Standard](#)
5. [ZymoBIOMICS Fecal Reference](#)
6. [hifiasm GitHub](#)
7. [hifiasm-meta GitHub](#)
8. [myloasm GitHub](#)
9. [HiFi-MAG-Pipeline](#)

Research use only. Not for use in diagnostic procedures. ©2026 Pacific Biosciences of California, Inc. ("PacBio"). All rights reserved. Information in this document is subject to change without notice. PacBio assumes no responsibility for any errors or omissions in this document. Certain notices, terms, conditions and/or use restrictions may pertain to your use of PacBio products and/or third-party products. Refer to the applicable PacBio terms and conditions of sale and to the applicable license terms at pacb.com/license. Pacific Biosciences, the PacBio logo, PacBio, Circulomics, Omniome, SMRT, SMRTbell, Iso-Seq, Sequel, Nanobind, Revio, Revio SPRQ, Kinnex, PureTarget, SPRQ, Vega and Vega SPRQ are trademarks of PacBio. Reference to third-party companies, products, or services are for informational purposes only and do not imply endorsement, sponsorship, or affiliation.

Appendix A. Complete sequencing metrics

The tables below show the general data metrics. Dashes indicate metrics that were not applicable or not reported for the corresponding workflow. Demultiplexing percentages are not directly comparable because pooling strategies differed across runs.

A1. Read length, read quality, SMRTbell barcoding, and yield

Workflow	Sample	System	Mean read length	Median Q	SMRTbell barcoded reads	% movie reads	Final HiFi yield
Standard HiFi	ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	6.32 kb	Q51	0.40 M	45.93%	2.52 Gb
Standard HiFi	ZymoBIOMICS Fecal Reference (D6323)	Vega	7.74 kb	Q47	4.18 M	4.38%	32.4 Gb
Standard HiFi	16 bacterial isolates	Vega	15.29 kb	Q35	4.22 M	99.53%	64.6 Gb
Ampli-Fi	ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	5.76 kb	Q50	4.53 M	49.69%	2.01 Gb
Ampli-Fi	ZymoBIOMICS Fecal Reference (D6323)	Vega	6.55 kb	Q48	4.53 M	49.69%	20.9 Gb
Ampli-Fi	16 bacterial isolates	Vega	7.98 kb	Q42	8.13 M	100%	57.4 Gb
seqWell LongPlex PCR+	ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	4.84 kb	Q48	11.43 M	99.84%	9.80 Gb
seqWell LongPlex PCR+	ZymoBIOMICS Fecal Reference (D6323)	Vega	5.38 kb	Q46	11.43 M	99.84%	49.8 Gb
seqWell LongPlex PCR+	16 bacterial isolates	Vega	5.93 kb	Q44	10.88 M	99.87%	62.3 Gb
seqWell LongPlex PCR-free	ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	5.15 kb	Q50	5.48 M	52.31%	3.52 Gb
seqWell LongPlex PCR-free	ZymoBIOMICS Fecal Reference (D6323)	Vega	5.63 kb	Q49	5.48 M	52.31%	13.8 Gb
seqWell LongPlex PCR-free	16 bacterial isolates	Vega	7.84 kb	Q46	4.20 M	50.82%	21.8 Gb

A2. Workflow-specific demultiplexing and duplicate metrics

Workflow	Sample	Ampli-Fi/LongPlex barcoded reads	% workflow demux reads	Unique reads	% duplicate reads	% unique reads	Final HiFi yield
Standard HiFi	ZymoBIOMICS Gut Microbiome Standard (D6331)	-	-	-	-	-	2.52 Gb
Standard HiFi	ZymoBIOMICS Fecal Reference (D6323)	-	-	-	-	-	32.4 Gb
Standard HiFi	16 bacterial isolates	-	-	-	-	-	64.6 Gb
Ampli-Fi	ZymoBIOMICS Gut Microbiome Standard (D6331)	0.41 M	9.17%	0.41 M	0.02%	99.98%	2.01 Gb
Ampli-Fi	ZymoBIOMICS Fecal Reference (D6323)	3.80 M	83.97%	3.79 M	0.16%	99.84%	20.9 Gb
Ampli-Fi	16 bacterial isolates	7.29 M	89.7%	7.29 M	0.007%	89.7%	57.4 Gb
seqWell LongPlex PCR+	ZymoBIOMICS Gut Microbiome Standard (D6331)	2.03 M	17.77%	2.02 M	0.34%	99.66%	9.80 Gb
seqWell LongPlex PCR+	ZymoBIOMICS Fecal Reference (D6323)	9.29 M	81.30%	9.26 M	0.35%	99.65%	49.8 Gb
seqWell LongPlex PCR+	16 bacterial isolates	10.82 M	99.5%	10.71 M	1.06%	98.4%	62.3 Gb
seqWell LongPlex PCR-free	ZymoBIOMICS Gut Microbiome Standard (D6331)	0.68 M	12.48%	-	-	-	3.52 Gb
seqWell LongPlex PCR-free	ZymoBIOMICS Fecal Reference (D6323)	2.45 M	44.66%	-	-	-	13.8 Gb
seqWell LongPlex PCR-free	16 bacterial isolates	2.97 M	70.6%	-	-	-	21.8 Gb

Appendix B. Bacterial isolate reference panel

ATCC strain information with ID, expected genome size, expected contig sizes, and GC content.

Bacterial isolate	ATCC ID	Expected genome size	Expected contig sizes	GC%
<i>Acinetobacter baumannii</i> strain AYE	BAA-1710D-5	3.96 Mb	3.9M; 9.7k; 5.6k; 2.7k	39.4%
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> strain CIP 702	33560D-5	1.77 Mb	1.79M	30.2%
<i>Clostridium perfringens</i>	13124D-5	3.26 Mb	3.26M	28.4%
<i>Cronobacter sakazakii</i> strain 2001-10-01	BAA-894D-5	4.51 Mb	4.3M; 131.2k; 31.2k; 2.7k	56.7%
<i>Enterobacter cloacae</i> subsp. <i>cloacae</i> strain CDC 442-68	13047D-5	5.61 Mb	5.3M; 199.6k; 86.3k; 5.1k	54.6%
<i>Enterococcus faecium</i> strain MMC4	51559D-5	3.16 Mb	2.8M; 215.9k; 63.7k; 43.0k; 12.5k; 4.4k; 2.9k; 2.0k	37.7%
<i>Escherichia coli</i> strain MG1655	700926D-5	4.64 Mb	4.6M	50.8%
<i>Escherichia coli</i> strain H10407	35401D-5	5.39 Mb	5.2M; 94.8k; 67.9k; 66.6k; 5.8k; 5.5k	50.7%
<i>Klebsiella pneumoniae</i> strain 1000527, 7561	BAA-2146D-5	5.78 Mb	5.4M; 140.8k; 117.8k; 84.4k; 2.0k	57.0%
<i>Listeria monocytogenes</i> strain Li 2	19115D-5	2.95 Mb	3.0M	38.0%
<i>Pseudomonas aeruginosa</i> strain PAO1-LAC	47085D-5	6.30 Mb	6.3M; 12.3k; 11.7k	66.5%
<i>Salmonella enterica</i> subsp. <i>Enterica</i> serovar Typhimurium strain LT2	700720D-5	4.95 Mb	4.9M; 93.9k	52.2%
<i>Shigella flexneri</i> strain 2457T	700930D-5	4.82 Mb	4.6M; 165.7k; 4.1k; 3.2k	50.7%
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> strain Seattle 1945	25923D-5	2.81 Mb	2.8M; 27.5k	32.9%
<i>Vibrio harveyi</i> strain BB120	BAA-1116D-5	6.07 Mb	3.8M; 2.2M; 89.0k	45.4%
<i>Vibrio parahaemolyticus</i> strain EB101	17802D-5	5.15 Mb	3.3M; 1.9M	45.3%