Optimizing PacBio sequencing: balancing cost, workflow simplicity, and input needs across HiFi, Ampli-Fi, and LongPlex PCR+ library prep methods

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Background

High-quality long-read sequencing has become essential for genome assembly, variant detection, and metagenomic profiling and assembly. While the PacBio HiFi workflow delivers industry-leading read accuracy and contiguity, researchers increasingly require solutions that reduce input requirements, simplify preparation, and lower per-sample cost. To address these needs, we evaluated three PacBio workflows: standard HiFi library preparation[1], the Ampli-Fi protocol[2], and the seqWell LongPlex[3] PCR+ method (Fig. 1) on representative genomic DNA samples including plant, microbial isolates, and metagenomic samples. Libraries were sequenced on the PacBio Revio and/or Vega systems (Fig. 2) and analyzed appropriately (Fig. 3). We assessed key metrics including library simplicity, sequence data quality, HiFi read length, HiFi data yield, barcoded reads, duplicate reads, genome assembly metrics, genome alignment coverage, per-sample reagent costs, and preparation time (Fig. 1,4; Tbl. 1-4). This comparison illustrates the flexibility of PacBio's HiFi sequencing ecosystem and quantifies trade-offs among quality results, input requirements, cost, and workflow simplicity. This study provides researchers with a practical framework for selecting the optimal PacBio workflow to suit their needs.

PacBio HiFi WGS library prep solutions

SMRTbell prep kit 3.0, HiFi prep kit 96, HiFi plex prep kit 96

- Benefits:
  - Maximum mean HiFi read lengths
  - Methylation information
  - Multiplexing up to 384
  - Prep time is ~3.5 – 6.5 hours*
- Limitations:
  - Higher library prep cost per sample (~$39 – $82 depending on prep kit used**)
  - Requires 50 - 500 ng gDNA per sample depending on plexity

Ampli-Fi protocol

- Benefits:
  - HiFi sequencing with as little as 1 ng of gDNA
  - Hi-C on HiFi with CiFi application
  - Access to challenging sample types
  - Multiplexing up to 384 x 96
  - Prep time is ~7 hours*
- Limitations:
  - Loss of methylation information
  - Slightly shorter mean HiFi read lengths
  - Higher library prep cost per sample (~$42 – $85 depending on prep kit used**)

seqWell LongPlex kit

- Benefits:
  - Shearing and barcoding in one step
  - Easier workflow and faster TAT (prep time is ~5.5 hours for 96 samples*)
  - Lowest library prep cost per sample (~$23 – $107 depending on plexity**)
  - Multiplexing up to 384 x 96
- Limitations:
  - If PCR+ approach, loss of methylation information
  - Shorter mean HiFi read lengths
  - For metagenomes/fragmented/lower MW DNA, PCR-free has low demux rate
  - Requires ≥150 ng gDNA per sample

Figure 1. HiFi WGS library prep solutions outlining benefits and limitations for each workflow. *Manual preparation time without using SRE. Time does not include optional QC checkpoints as that will vary by lab equipment used. **All prices are listed in USD and cost may vary by region. Pricing includes library reagents and does not include instrument amortization, other reagents, or DNA extraction.

Experimental design

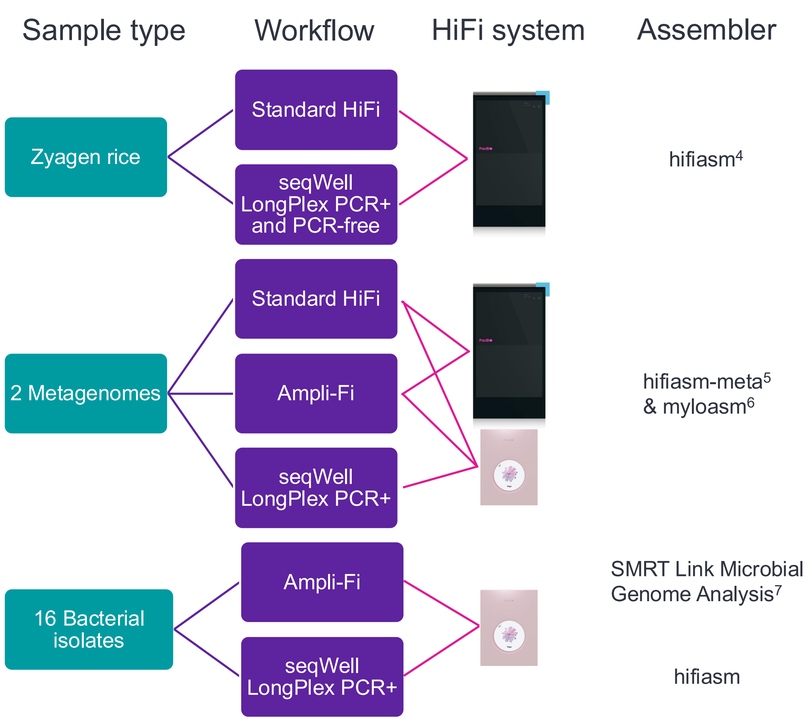

Figure 2. Study experimental design with sample type, library prep workflow, HiFi system, and assembler used on HiFi data. All Revio and Vega runs were 24 hours. The assembler used for bacterial isolates differs between the two workflows for this study.

Analysis workflows

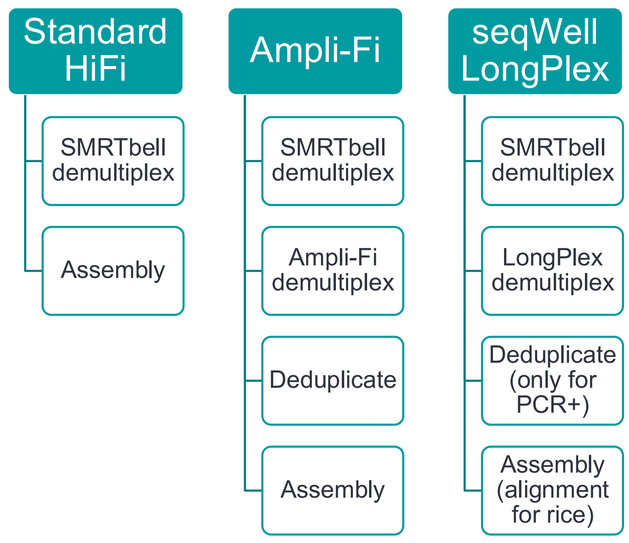

Figure 3. Analysis steps for the different library prep workflows.

Data metrics

Sample	Instrument	Read length (mean)	Read quality (median)	SMRTbell barcoded reads	Ampli-Fi or seqWell barcoded reads	Unique reads	Duplicate reads	Final yield
Standard HiFi prep								
Zyagen rice	Revio	7.86 kb	Q40	3.34 M	–	–	–	26.24 Gb
ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	6.32 kb	Q51	0.40 M	–	–	–	2.52 Gb
ZymoBIOMICS Gut Microbiome Standard (D6331)	Revio	6.90 kb	Q47	0.45 M	–	–	–	3.10 Gb
ZymoBIOMICS Fecal Reference (D6323)	Vega	7.74 kb	Q47	4.18 M	–	–	–	32.37 Gb
ZymoBIOMICS Fecal Reference (D6323)	Revio	8.74 kb	Q44	5.01 M	–	–	–	43.80 Gb
Ampli-Fi								
ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	5.76 kb	Q43	–	0.41 M	0.41 M	0.02%	2.39 Gb
ZymoBIOMICS Gut Microbiome Standard (D6331)	Revio	6.30 kb	Q41	–	0.47 M	0.47 M	0.02%	2.95 Gb
ZymoBIOMICS Fecal Reference (D6323)	Vega	6.56 kb	Q42	–	3.80 M	3.79 M	0.16%	24.90 Gb
ZymoBIOMICS Fecal Reference (D6323)	Revio	7.43 kb	Q41	–	5.14 M	5.12 M	0.19%	38.05 Gb
16 Bacterial isolates x 6 replicates	Vega	7.88 kb	Q42	–	7.29 M	7.29 M	0.007%	57.43 Gb
seqWell LongPlex								
PCR-free: Zyagen rice x 24 replicates	Revio	5.11 kb	Q44	2.73 M	1.46 M	–	–	7.45 Gb
PCR+: Zyagen rice x 24 replicates	Revio	5.70 kb	Q43	5.34 M	4.76 M	4.76 M	0.01%	27.14 Gb
PCR+: ZymoBIOMICS Gut Microbiome Standard (D6331)	Vega	4.84 kb	Q41	11.43 M	2.03 M	2.02 M	0.30%	9.80 Gb
PCR+: ZymoBIOMICS Fecal Reference (D6323)	Vega	5.38 kb	Q41		9.29 M	9.26 M	0.30%	49.72 Gb
PCR+: 16 Bacterial isolates	Vega	5.82 kb	Q40	10.88 M	10.82 M	10.71 M	1.10%	62.32 Gb

Table 1. General data metrics for different library prep workflows, sample types, and PacBio instruments from this study.

Assembly results

a

Standard HiFi prep assembly	Size	Contigs	N50	L50
Primary	400.0 Mb	654	30.2 Mb	6
Alternate	17.7 Mb	636	31.4 kb	205

b

seqWell LongPlex	>1x coverage	>2x coverage	>5x coverage	Mean reads per barcode	Mean duplicate reads per barcode
PCR-free	18.12%	5.40%	0.23%	60.8 k	N/A
PCR+	70.09%	48.71%	9.53%	198.4 k	19.7

Table 2. a) Standard HiFi prep assembly results for rice sample run on the Revio system. b) seqWell LongPlex PCR-free and PCR+ alignment to standard HiFi prep assembly coverage, mean reads/barcode, and mean duplicate reads/barcode.

Sample	sc-HQ-MAGs	mc-HQ-MAGs	MQ-MAGs	Total MAGs	Data (Gb)	Assembly
ZymoBIOMICS Gut Microbiome Standard (D6331)						
Standard HiFi prep	10	2	2	14	2.5	hifiasm
Ampli-Fi	8	3	0	11	2.0	hifiasm
seqWell LongPlex PCR+	7	2	2	11	2.0	hifiasm
Standard HiFi prep	7	2	4	13	2.5	myloasm
Ampli-Fi	8	3	0	11	2.0	myloasm
seqWell LongPlex PCR+	7	4	0	11	2.0	myloasm
ZymoBIOMICS Fecal Reference (D6323)						
Standard HiFi prep	42	31	134	207	24.7	hifiasm
Ampli-Fi	33	14	113	160	20.9	hifiasm
seqWell LongPlex PCR+	19	18	102	139	20.9	hifiasm
Standard HiFi prep	45	46	131	222	24.7	myloasm
Ampli-Fi	33	42	122	197	20.9	myloasm
seqWell LongPlex PCR+	19	47	105	171	20.9	myloasm

Table 3. Metagenome-assembled genome (MAG) results from the Vega system for the Zymo gut microbiome standard and fecal reference, each workflow, and two assemblers. Data for each sample was downsampled to ~ match the lowest number of reads.

sc-HQ-MAGs: single-contig high-quality MAGs (>90% completeness, <5% contamination); mc-HQ-MAGs: multiple-contig high-quality MAGs; MQ-MAGs: medium-quality MAGs (>50% completeness, <10% contamination).

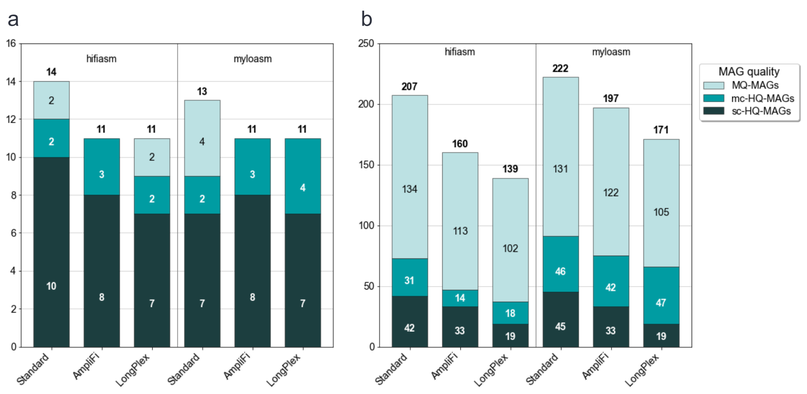

Figure 4. MAG results from the Vega system for the two metagenome samples, each workflow, and two assemblers adapted from Table 3. a) Zymo gut microbiome standard; b) Zymo pooled human fecal reference.

Assembly results continued

a

Bacterial isolate	ATCC ID	Genome size (Expected)	Contigs (Expected)	Circular (Expected)	GC%
Acinetobacter baumannii strain AYE	BAA-1710D-5	3.96 Mb	3.9M; 9.7k; 5.6k; 2.7k	yes; yes; yes; yes	39.35%
Campylobacter jejuni subsp. jejuni strain CIP 702	33560D-5	1.77 Mb	1.79M	yes	30.22%
Clostridium perfringens	13124D-5	3.26 Mb	3.26M	yes	28.38%
Cronobacter sakazakii strain 2001-10-01	BAA-894D-5	4.51 Mb	4.3M; 131.2k; 31.2k; 2.7k	yes; yes; yes; yes	56.73%
Enterobacter cloacae subsp. cloacae strain CDC 442-68	13047D-5	5.61 Mb	5.3M; 199.6k; 86.3k; 5.1k	yes; yes; yes; no	54.58%
Enterococcus faecium strain MMC4	51559D-5	3.16 Mb	2.8M; 215.9k; 63.7k; 43.0k; 12.5k; 4.4k; 2.9k; 2.0k	yes; yes; yes; yes; yes; yes; yes; yes	37.71%
Escherichia coli strain MG1655	700926D-5	4.64 Mb	4.6M	yes	50.79%
Escherichia coli strain H10407	35401D-5	5.39 Mb	5.2M; 94.8k; 67.9k; 66.6k; 5.8k; 5.5k	yes; yes; yes; yes; yes; yes	50.71%
Klebsiella pneumoniae strain 1000527, 7561	BAA-2146D-5	5.78 Mb	5.4M; 140.8k; 117.8k; 84.4k; 2.0k	yes; yes; yes; yes; yes	56.97%
Listeria monocytogenes strain Li 2	19115D-5	2.95 Mb	3.0M	yes	37.99%
Pseudomonas aeruginosa strain PAO1-LAC	47085D-5	6.30 Mb	6.3M; 12.3k; 11.7k	yes; yes; yes	66.51%
Salmonella enterica subsp. Enterica serovar Typhimurium strain LT2	700720D-5	4.95 Mb	4.9M; 93.9k	yes; yes	52.24%
Shigella flexneri strain 2457T	700930D-5	4.82 Mb	4.6M; 165.7k; 4.1k; 3.2k	yes; yes; yes; yes	50.74%
Staphylococcus aureus subsp. aureus strain Seattle 1945	25923D-5	2.81 Mb	2.8M; 27.5k	yes; yes	32.86%
Vibrio harveyi strain BB120	BAA-1116D-5	6.07 Mb	3.8M; 2.2M; 89.0k	yes; yes; yes	45.43%
Vibrio parahaemolyticus strain EB101	17802D-5	5.15 Mb	3.3M; 1.9M	yes; yes	45.33%

b

	Ampli-Fi	seqWell LongPlex PCR+
Perfect assemblies	7	3
	Clostridium perfringens **Cronobacter sakazakii strain 2001-10-01* *Escherichia coli strain MG1655* *Listeria monocytogenes strain Li 2* *Salmonella enterica subsp. Enterica serovar Typhimurium strain LT2* *Staphylococcus aureus subsp. aureus strain Seattle 1945* *Vibrio parahaemolyticus strain EB101*	*Escherichia coli strain MG1655* *Listeria monocytogenes strain Li 2* *Vibrio parahaemolyticus strain EB101*
Near-perfect assemblies	1	2
	Campylobacter jejuni subsp. jejuni strain CIP 702	*Campylobacter jejuni subsp. jejuni strain CIP 702* *Salmonella enterica subsp. Enterica serovar Typhimurium strain LT2*

Table 4. a) Bacterial isolate references and b) assembly results. The Ampli-Fi run had 6 replicates of each of the 16 isolates for a 96-plex run on the Vega system and the assembly results are based on one representative replicate. The seqWell LongPlex run had 16 isolates with no replicates on the Vega system and the HiFi data was downsampled to simulate ~ a 192-plex (~200 Mb/sample) and then assembled. seqWell LongPlex assembly results are based on downsampled data. Perfect assemblies: single-contig chromosome(s) and all are circular; Near-perfect assemblies: single-contig chromosome(s), but not all are circular. *Perfect except missing smallest contig due to library size selection.

Summary

- **Standard HiFi library preparation** provided the **highest quality results** across all evaluated metrics.
- **Ampli-Fi** supported the **lowest DNA input amounts** and delivered higher quality results than LongPlex (but lower than standard HiFi prep), while still enabling pooling prior to library preparation.
- **seqWell LongPlex** achieved the **lowest per-sample cost** depending on plexity and offered the **simplest workflow** via streamlined shearing and and upfront barcoding, though with reduced quality results relative to the other two approaches.
- For the rice sample, **seqWell LongPlex PCR-free retained DNA methylation** profiles comparable to standard HiFi libraries; however, low barcode recovery limited genome coverage. **PCR+** libraries yielded **substantially improved coverage**, indicating the need for further PCR-free optimization.
- For the pooled human fecal reference metagenome sample, **standard HiFi prep** generated the **highest number of MAGs**, followed by Ampli-Fi and seqWell LongPlex PCR+. **Ampli-Fi and LongPlex PCR+** performed similarly for the mock community, producing **equivalent numbers of high-quality MAGs**.
- For bacterial isolates, **Ampli-Fi** produced **more perfect and near-perfect genome assemblies** than seqWell LongPlex PCR+.
- **All workflows** achieved **per-sample costs below $100 USD**, depending on genome size and/or multiplexing, enabling high-throughput applications such as long-read low-pass genotyping, metagenome assembly and profiling, and microbial isolate assembly.

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