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Application note

Designing long-range PCR amplicons for HiFi sequencing

Amplicon sequencing is a widely used targeted sequencing approach for applications such as rare disease research, inherited disease testing, pharmacogenomics, HLA typing, and characterization of clinically relevant genomic loci. By focusing sequencing on predefined regions of interest, it enables deep, high-confidence analysis while simplifying data interpretation and reducing sequencing requirements. Amplicon-based approaches are particularly valuable when complete characterization of a specific gene or locus is needed, including comprehensive detection of sequence variation and accurate haplotype resolution.

Long-range PCR (LR-PCR) extends these capabilities by amplifying substantially larger genomic regions than conventional PCR, enabling contiguous amplicons of approximately 5–25 kb that are ideal for long-read sequencing, with larger amplicons achievable through optimized assays. Rather than interrogating a target using multiple short overlapping fragments, LR-PCR captures an entire gene, haplotype block, or other genomic interval in one or a small number of amplicons. This preserves the long-range genomic context needed to characterize multiple variants together across a continuous region while reducing assay complexity.

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PacBio® HiFi sequencing is particularly well suited to LR-PCR amplicons because its long, highly accurate reads frequently span the entire amplified fragment. Sequencing complete amplicons as single reads enables comprehensive characterization of the target region without the assembly or phasing challenges associated with shorter reads.

HiFi sequencing provides several advantages for LR-PCR amplicons:

- **Complete amplicon coverage** — HiFi reads often span the entire amplified fragment, eliminating the need for local assembly.
- **High read accuracy** — HiFi reads achieve >99.9% accuracy, enabling confident detection of SNVs and small insertions and deletions.
- **Direct haplotype phasing** — Variants within the same amplicon can be assigned to individual alleles using read-backed phasing.
- **Comprehensive variant detection** — A single assay can identify SNVs, indels, structural variants, and copy number changes within the amplified region.
- **High-throughput multiplexing** — Hundreds of barcoded amplicons can be pooled into a single sequencing run, supporting efficient targeted studies.
- **Scalable targeted workflows** — Applicable to projects ranging from single-gene analysis to multiplexed gene panels.

For many targeted sequencing applications, HiFi sequencing of LR-PCR amplicons can simplify laboratory workflows by consolidating multiple analyses into a single sequencing assay. Instead of combining several complementary methods to fully characterize a target region, a single HiFi sequencing workflow can provide comprehensive sequence information, direct haplotype phasing, structural variant characterization, repeat sizing, and other genomic insights from the same long-range amplicon (Table 1).

This application note provides practical guidance for designing long-range PCR amplicons optimized for HiFi sequencing, including recommendations for primer design, assay optimization, library preparation, sequencing, and data analysis.

How HiFi sequencing consolidates multiple analytical methods

For many genomic targets, conventional approaches require a combination of multiple specialized assays: Sanger sequencing for point mutations, MLPA or array CGH for copy number variants, Southern blotting for repeat expansions, or repeat primed-PCR (RP-PCR) for triple-repeat disorders, and linkage analysis for phasing. HiFi sequencing with LR-PCR amplicons can consolidate all of these assays in a single integrated workflow (Table 1) and delivers full sequence, phased haplotypes, structural variants, repeat sizes, methylation status, and SNVs, all from a single library prep and sequencing run.

Legacy assay	Limitation	Replaced by HiFi LR-PCR
Sanger sequencing	Short reads, no phasing, slow	Full-length amplicon, direct phasing
MLPA/Array CGH	Only detects copy number, no sequence	CNV + sequence + breakpoint resolution
Southern blot	Low resolution, qualitative	Exact repeat length, allele-specific
RP-PCR	Detects expansión, cannot size accurately	Precise repeat number, sequence context
Short-read NGS panel	Cannot phase, misses SVs, dark regions	Full variant spectrum, phased, complete
Statistical haplotyping	Probabilistic, not definitive	Direct physical haplotypes from reads

Table 1. Methods replaced or consolidated by HiFi amplicon sequencing in a single workflow.

Step-by-step: Designing LR-PCR primers for HiFi sequencing

The workflow on the following page follows the four-stage framework established in the peer-reviewed primer design literature (Thornton & Basu, 2011; Bustin et al., 2017) and adapts it specifically to the requirements of long-range amplification and HiFi sequencing.

Long-range PCR amplicon workflow for HiFi sequencing

STEP 1

Define your target region

Retrieve the reference sequence for your locus from NCBI or Ensembl. Identify the full genomic coordinates including relevant exons, introns, and regulatory regions. For clinical loci, check for known variants in ClinVar and gnomAD to avoid placing primers over polymorphic sites. Define amplicon boundaries to capture the complete region of interest within 5–15 kb.

STEP 2

Check template complexity: GC content and secondary structure

Use UCSC Genome Browser or the EMBOSS Geecee tool to assess the GC profile across the amplicon. Avoid priming within or across regions with >70% or <30% GC content. Use Mfold or IDT OligoAnalyzer to check for strong secondary structures at the intended primer binding sites. For GC-rich templates (e.g., trinucleotide repeats), select primer sites flanking but not within the repeat tract.

STEP 3

Design primer pairs using Primer-BLAST or Primer3

Use NCBI Primer-BLAST or Primer3 to generate candidate primers flanking your target. Input the specific long-range parameters described below. Enable BLAST specificity checking against the whole genome to exclude primers with off-target binding. For degenerate templates or highly polymorphic populations, also run primers through the 1000 Genomes variation track in Ensembl Variant Effect Predictor.

STEP 4

Validate primer properties in silico

Paste each primer into IDT OligoAnalyzer and check: (a) T_m using nearest-neighbor thermodynamics; (b) self-complementarity / hairpin structures (ΔG should be > −3 kcal/mol); (c) homodimer and heterodimer potential. Both primers in a pair must have T_m values within 2–3°C. For LR-PCR, the NEB T_m Calculator matched to your chosen polymerase is strongly recommended.

STEP 5

Select a high-fidelity polymerase

Long-range amplification requires a proofreading polymerase with high processivity and error correction. Standard Taq DNA polymerase is NOT suitable for LR-PCR. Choose from the recommended polymerases described below, and calculate your final annealing temperature using the polymerase manufacturer's T_m calculator. The extension time must be calibrated to amplicon length, typically 1–2 min/kb.

STEP 6

Optimize & test the PCR reaction

Run gradient PCR across a range of annealing temperatures (typically Tm −5°C to Tm +2°C). Assess amplification by agarose gel electrophoresis (0.5–0.8% agarose for large fragments). A single, clean band at the expected size is essential. Troubleshoot with DMSO (0.4–2%), betaine (0.5–3.0 M), or by adjusting Mg^{2+} concentration (1.5–4 mM). Quantify the amplicon with Qubit dsDNA BR Assay (Thermo Fisher) before library prep.

STEP 7

Prepare HiFi library and sequence

Use the PacBio SMRTbell Prep Kit 3.0 or HiFi Plex Prep Kit 96 for amplicon library preparation. For multiplexed runs, add barcoded adapters during library preparation. Optimal input is a clean, single-band amplicon of 5–25 kb. Sequence on PacBio Revio® or Vega™ systems. For amplicons up to 15 kb, full-length HiFi reads covering the entire amplicon are routinely obtained.

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Parameter	Standard PCR	Long-range PCR	Rationale
Primer length	18–22 bp	22–30 bp	Greater length increases T_m and specificity on large templates
GC content	40–60%	40–50%	Lower GC ceiling reduces secondary structure in long primers
Melting temperature (T_m)	50–65°C	58–68°C	Higher T_m needed for specificity across large amplicons
Tm difference (fwd vs rev)	<5°C	<3°C (ideally <2°C)	Tight T_m matching is critical for balanced long-range extension
Annealing temperature (T_a)	T_m −5°C	T_m −3°C to Tm +1°C	Higher T_a reduces non-specific priming on complex templates
3' GC clamp	1–2 G/C bases	1–2 G/C bases	Stabilizes extension initiation; avoid ≥3 consecutive G/C
3' terminal base	G or C preferred	G or C; avoid 3'-T	3'-T destabilizes polymerase binding and reduces efficiency
Primer concentration	0.2–0.4 µM	0.1–1.0 µM (optimize)	Lower for complex templates; up to 1 µM for 17+ kb targets
Runs of single base	Avoid ≥4	Avoid ≥4	Homopolymer runs cause slippage and mispriming
Repetitive elements	Avoid	Must avoid	Primers spanning repeats cause multiple non-specific products
3' self-complementarity ΔG	> −3 kcal/mol	> −3 kcal/mol	Critical — primer dimers block long-range extension completely
Extension time	1 min/kb	1–2 min/kb	Allow full-length synthesis; longer for difficult templates
Denaturation temperature	94°C / 30 s	93°C / 15–20 s	Shorter denaturation reduces polymerase degradation
Number of PCR cycles	30–35	25–30	Fewer cycles reduce chimera and artifact formation
Template DNA quality	Standard	High molecular weight, intact	Sheared or degraded DNA will not yield long amplicons

Table 2. Recommended primer design parameters for long-range PCR (>5 kb amplicons) calibrated for HiFi sequencing.

Primer Design Parameters for LR-PCR

The parameters above are specifically calibrated for long-range amplification (>5 kb). They are derived from the QIAGEN LongRange PCR Handbook, the Takara Bio PrimeSTAR LongSeq Technical note, the primer design guidelines from Bustin et al. (2017), and the New England Biolabs primer design guidance.

Note: LR-PCR is highly sensitive to template DNA quality. A broken or nicked template cannot serve as a substrate for long-range extension. Always use high-molecular-weight (HMW) DNA extracted using gentle lysis protocols (e.g., PacBio Nanobind® kits, magnetic bead-based extraction or salting-out methods). Verify integrity by pulse-field gel or Femto Pulse analysis before proceeding. Input DNA should show a predominant band >30 kb.

Sample multiplexing

Indexed primers and adapters

If more than 384 samples per SMRT® Cell are required, A combination of indexed primers and indexed SMRTbell® adapters may be used. Uniquely indexed amplicons are pooled in a PacBio HiFi library prepared with indexed SMRTbell adapters. Using the same set of indexed primers for each indexed SMRTbell adapter, HiFi libraries may then be pooled and sequenced on one SMRT Cell. Two rounds of demultiplexing is required: round 1 demultiplexes SMRTbell adapter indices, and round 2 demultiplexes primer indices.

PCR amplicon-based HiFi target enrichment with indexed primers

A streamlined and cost-effective targeted sequencing approach is PCR with barcoded primers (Figure 1). Consult the Application note and protocol for more details. Recommended barcode sequences to support 384 dual-indexed samples are available.

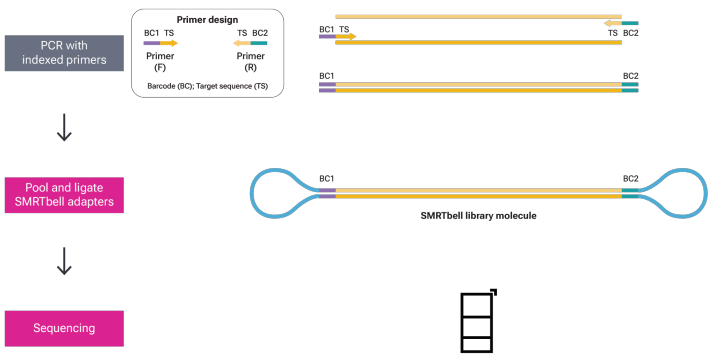

Figure 1: Target-specific primers with barcode tails (purple and teal) are used to amplify and index a region of interest. Samples are pooled and a single PacBio library is prepared for sequencing.

PCR amplicon-based target enrichment with indexed adapters

A streamlined targeted sequencing approach for existing PCR assays is to use indexed SMRTbell adapters. With the automated HiFi plex prep kit, prepare individual SMRTbell libraries for each sample, pool, and sequence on one SMRT Cell. Consult the PacBio protocol for more details.

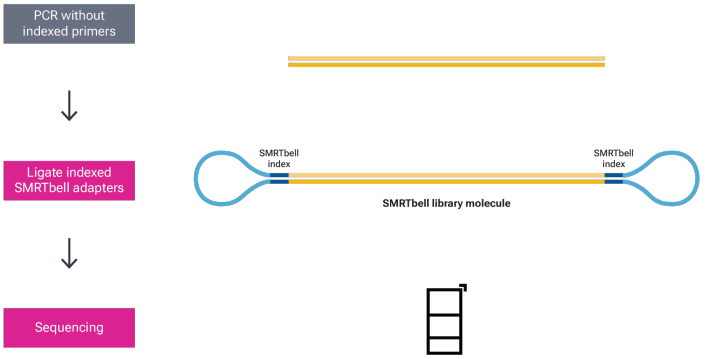

Figure 2: Target-specific primers are used to amplify a region of interest. Barcode sequences (dark blue) are added during HiFi library prep.

PCR amplicon-based target enrichment with M13 indexed primers

A flexible and cost-effective targeted sequencing approach is to add indices with PCR and barcoded M13 primers. Consult the PacBio protocol, the barcoded FASTA file for demultiplexing, and the M13 primer plate layout for more information.

Figure 3: Target-specific primers with M13 tails are used to amplify a region of interest. Dual indices (pink and teal) are added with barcoded M13 primers. Samples are pooled and a single PacBio library is prepared for sequencing.

Recommended polymerases for LR-PCR

The choice of DNA polymerase is the most critical variable in long-range PCR. A mixture of a highly processive *Taq* variant and a proofreading polymerase (such as *Pwo* or *Pyrococcus*-based enzymes) in a ratio of approximately 10:1 to 20:1 (*Taq*:proofreader) is the classical approach. Blended formulations available commercially are strongly preferred for their convenience and optimized ratios.

Recommended: Takara PrimeSTAR LongSeq DNA Polymerase

The Takara Bio PrimeSTAR LongSeq DNA Polymerase is specifically formulated for long-range amplification and is the primary recommendation for use with HiFi sequencing due to its extremely high fidelity and processivity. Consult the Takara Bio Technical note for more detailed primer design guidance specific to this enzyme.

- Amplicon capacity: Up to 35 kb (genomic DNA)
- Fidelity: High-fidelity with 3' to 5' exonuclease proofreading
- T_m requirement: Primers should have higher-than-standard Tm due to the enzyme's high specificity
- Protocol: Uses a two-step PCR cycle (combined annealing/extension) optimized per amplicon length

Polymerase	Manufacturer	Max Amplicon	Key Feature	Best For
PrimeSTAR LongSeq	Takara Bio	35 kb	Exceptional fidelity + processivity	Primary recommendation for HiFi prep
DeCodiFi™ Long& Complex	Kura Biotech	30 kb	Hot-start high-fidelity 2X all-in-one mix optimized for long, GC-rich, repetitive/expansion-repeat, and low-input DNA	Difficult-template PCR, high-GC/repeat regions, low-input samples
KOD Xtreme™ Hot Start DNA Polymerase	Millipore Sigma	24 kb (gDNA)	10X higher fidelity than *Taq* blends, eliminates mispriming and primer-dimer formation	amplifies challenging DNA templates with up to 90% GC content
LA Taq (Long and Accurate)	Takara Bio	48 kb	Blended Taq + proofreader; robust	Genotyping, routine LR-PCR
KAPA HiFi HotStart	Roche / KAPA	30+ kb	Superior GC tolerance	GC-rich loci, high specificity panels
Q5 Hot Start High-Fidelity	NEB	20+ kb	Lowest error rate for its class	Amplicons requiring minimum errors
PrimeSTAR GXL	Takara Bio	30 kb	Handles difficult, complex templates	Challenging loci, secondary structure
Platinum SuperFi II	Thermo Fisher	20+ kb	Universal 60°C annealing temp	Rapid protocol development
Advantage 2 Polymerase Mix	Takara Bio	30+ kb	High-throughput genotyping	Large-scale screening projects

Table 3. Recommended high-fidelity polymerases for LR-PCR amplicon preparation prior to HiFi sequencing.

Alternative polymerases

The alternative polymerases listed above are also verified for LR-PCR and may be preferred depending on amplicon size, GC content, or available laboratory infrastructure. Note for amplicons 5–15 kb intended for direct HiFi sequencing, Takara PrimeSTAR LongSeq or KAPA HiFi HotStart are preferred because their low error rates minimize background variants in the sequencing data. For amplicons >15 kb, Takara LA Taq or PrimeSTAR GXL offer the processivity required for successful amplification.

Optimizing thermal cycling conditions

Thermal cycling conditions play a major role in amplification success. Long-range PCR generally benefits from gentler cycling conditions than conventional short-amplicon PCR. As a starting point, follow thermal cycling conditions recommended by the manufacturer. For example, the PrimeSTAR LongSeq DNA Polymerase may work better with a 3-step PCR instead of a 2-step cycling condition depending on the primer length.

Practical guidance is to:

- **Use 3-step first** when primers anneal around **55–64°C** or are still optimizing.
- **Use 2-step** when primers can anneal at **~68–72°C**, or when a long/GC-rich target produces smear or nonspecific bands under 3-step conditions.

Two-step conditions		Three-step conditions		
Temperature	Time	Temperature	Time	
94°C	1 min	94°C	1 min	
98°C	10 sec	98°C	10 sec	30 cycles
68°C	30 sec/kb	57°C	15 sec	
		68°C	30 sec/kb	

Table 4. PCR cycling conditions for two- and three- step PCR. The PCR protocol allows for different annealing and extension temperatures.

A typical long-range PCR may include:

Step	Temperature	Time
Initial denaturation	98°C	30 sec
Denaturation	98°C	10 sec
Annealing	60–68°C	15–30 sec
Extension	68°C	30–60 sec/kb
Final extension	68°C	5–10 min

Table 5. Typical long-range PCR protocol conditions.

Extension time should scale proportionally with amplicon size. For example:

- 2 kb targets may require 1–2 minutes
- 5 kb targets may require 3–5 minutes
- 10 kb targets may require 7–10 minutes
- 15 kb targets may require 12–15 minutes

Longer extension times often improve full-length product yield and reduce incomplete amplification products.

Cycle number should also be carefully controlled. Excessive cycling can increase chimera formation, introduce bias, and reduce overall library quality. In most cases, 23–32 cycles are sufficient.

DNA input mass

The total amount of DNA input required for constructing the SMRTbell library is dependent on the mean size of the amplicons.

Pre-indexed samples can be pooled prior to library prep to satisfy the DNA input mass requirements (see Table 6). The mass required per sample can be calculated by dividing the mass required from Table 6 by the number of samples per indexed pool. Refer to the table below for the minimum required input DNA mass into library preparation per SMRT Cell for different insert sizes.

It is recommended to pool amplicons by the bins specified in Table 1 to achieve optimal loading on PacBio sequencers. When amplicons are similar in size, it is acceptable to pool by mass for each sample. If the amplicons differ in size, pool by molarity to ensure even coverage. A pooling calculator is available in SMRT® Link under Sample Setup > Add calculation > Loading calculator.

Mean size	Revio SRPQ, Revio SPRQ-Nx, Vega SPRQ-Nx
1–3 kb	50 ng*
3–5 kb	100 ng
5–10 kb	200 ng
>10 kb	300 ng

Table 6. Recommended minimum pooled DNA input mass, binned by size, into library preparation for 1 SMRT Cell. *Note On Revio platforms, <5 kb libraries are not supported for SMRT Cell multi-use.

Recommended bioinformatics tools and databases

Consult tools and databases linked throughout this Application note. All links were verified at time of publication.

A practical bioinformatic workflow for long-range PCR amplicons should follow:

- CCS/HiFi read generation → demultiplexing → either pbAA clustering for phased consensus or reference mapping/variant calling for standard variant detection.
- For single clean amplicons, use CCS + demultiplexing + HiFi Mapping/variant calling. For *HLA*, *CYP2D6*, paralogous genes, pseudogenes, mixed alleles, or large indels, use pbAA. For repeat expansions, TRGT can be useful because it performs targeted tandem-repeat genotyping and visualization from HiFi data.

Problem	Likely cause	Recommended solution
No amplification	Poor template integrity or primer T_m mismatch	Check HMW DNA quality; lower T_a by 2°C; increase extension time
Smear/multiple bands	Non-specific priming; primer dimer formation	Increase T_a; use hot-start polymerase; reduce primer concentration
Faint/low-yield band	Insufficient template or short extension	Increase template to 100–200 ng; extend at 1.5 min/kb
GC-rich amplicon fails	Template secondary structure during denaturation	Add 0.4–1.0 µL DMSO or 0.5–3.0 M betaine; use KAPA HiFi
Correct band + smear	Over-cycling generating artefacts	Reduce cycle number to 25–28; use fewer cycles with more template
HiFi reads truncated	Amplicon input too short for adapter ligation	Ensure amplicon >500 bp; size-select >3 kb input for best results
High error rate in reads	Low-fidelity polymerase used in PCR	Switch to PrimeSTAR LongSeq or KAPA HiFi; reduce PCR cycles

Table 7. Common LR-PCR problems and solutions.

References

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular Detection and Quantification*, *14*, 19-28. https://doi.org/10.1016/j.bdq.2017.11.001

Thornton, B., & Basu, C. (2011). Real-time PCR (qPCR) primer design using free online software. *Biochemistry and Molecular Biology Education*, *39*(2), 145-154. https://doi.org/10.1002/bmb.20461

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