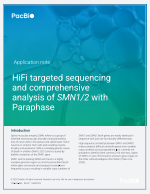
Technical note

SMA PCR ASSAY DEVELOPMENT

Note: This technical note, including the description of the PCR assay, is intended as an example guidance for the development of an amplicon assay for use on PacBio systems, and is neither optimized nor supported by PacBio.



Also available from PacBio: Application note — *HiFi targeted sequencing and comprehensive analysis of SMN1/2 with Paraphase*

Target region

SMN1 coordinates: chr5: 70,917,000–70,960,000 (hg38; 43,001 nt). These coordinates cover the entire *SMN1* gene plus upstream and downstream regions and, by extension, the homologous *SMN2* gene and surrounding regions. The coordinates were chosen to provide optimal coverage for identifying SMA mutations and carrier types, especially potential silent carriers based on haplotypes.

Amplicon requirements

A minimum of three amplicons is required to provide adequate coverage across these highly homologous genes. It is recommended for amplicons to be as long as possible with the greatest amount of overlap. Amplicons should be no longer than 25 kb to ensure optimal CCS read generation.

Increasing the number of amplicons allows for a decrease in size while maintaining adequate overlapping coverage. However, this potentially requires more effort in design optimization. While shorter amplicons of approximately 18 kb may require 24 hours of sequencing time to generate at least Q30 median HiFi read quality, amplicons of 25 kb may require 30 hours of sequencing time to generate the same median HiFi read quality.

Consideration should be given to whether these amplicons are to be co-amplified in a single PCR reaction. If so, designing the amplicons to be slightly different sizes will allow for yield balance determination by capillary or agarose gel electrophoresis. If the amplicons are amplified individually, they can be equimolarly pooled prior to library construction.

Primer design

Primer3Plus online design software is recommended for primer design (primer3plus.com). Primer melting temperature should match recommendations for the long-PCR polymerase or master mix that is to be used.

When designing primers, keep in mind that regions of homology, SNPs, and low-complexity sequence should be avoided. Multiple primer designs from a specific priming location should be tested empirically to find the best design, as *in silico* predictions can be inaccurate.

The use of barcoded primers paired asymmetrically generally allows for a more streamlined library construction workflow, as samples can be pooled before library construction. The tradeoff with using barcoded adapters is the need to maintain stocks of differently barcoded primers.

PCR chemistry

Testing several long-PCR enzymes or master mixes from different suppliers, if possible, usually helps shorten development time. Invitrogen Platinum SuperFi II PCR Master Mix from Thermo Fisher Scientific and LA Taq DNA polymerase HS from Takara Bio are good starting options. Ensure that hot-start enzymes are used for the best specificity possible.

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PCR condition optimizations

Follow optimization guidelines from the long-PCR enzyme or master mix supplier for template input amount, primer concentrations, and cycling conditions. Use of a PCR cycler with thermal gradient capabilities is helpful for quickly determining optimal primer annealing temperatures. To minimize formation of heteroduplexed amplicons, primer concentrations should not be limiting. Cycle numbers should be kept to a minimum value (typically under 30 cycles) that still provide adequate yield to continue with PCR reaction cleanup and QC and library construction. Optimal primer concentrations and cycle numbers should be determined empirically.

Note that the addition of PCR additives can greatly improve performance. Addition of betaine or DMSO or a combination of both have been found to improve specificity and yield with longer templates that may be difficult to keep denatured during primer annealing and extension. Suggested titration ranges are 0.25–3.0 M betaine in 0.25 M increments and 1–10% DMSO in 1% increments.

Questions? Visit **pacb.com/contact** or contact your local customer service representative

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