Technical overview

Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0

For targeted HiFi sequencing on PacBio long-read systems

Sequel II and IIe systems ICS v11.0
Vega system ICS v1.1
Revio system ICS v13.3
SMRT Link v25.3

PN 103-705-200 Rev 01 | November 2025

Technical overview

Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0

1. Multiplexed amplicon library preparation workflow overview
2. Multiplexed amplicon library preparation workflow details
3. Multiplexed amplicon library sequencing preparation workflow details
4. Multiplexed amplicon data analysis recommendations for variant detection applications
5. Library preparation workflow overview for target enrichment sequencing using Twist Bioscience hybrid capture panels
6. Library preparation workflow overview for target enrichment sequencing using QIAGEN hybrid capture panels
7. General recommendations for hybrid capture HiFi library preparation & sequencing
8. Technical documentation & applications support resources

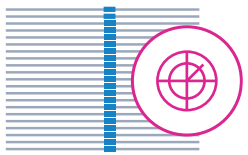
Advantages of targeted HiFi sequencing

Robust coverage of difficult-to-sequence or difficult–to-map regions

Unambiguous haplotype resolution through direct phasing without the need for trios

Ancestry-agnostic discovery of rare or novel variants

Custom panels, flexible scaling, cost-effective projects



High-accuracy variant calling including structural variants and copy number variation

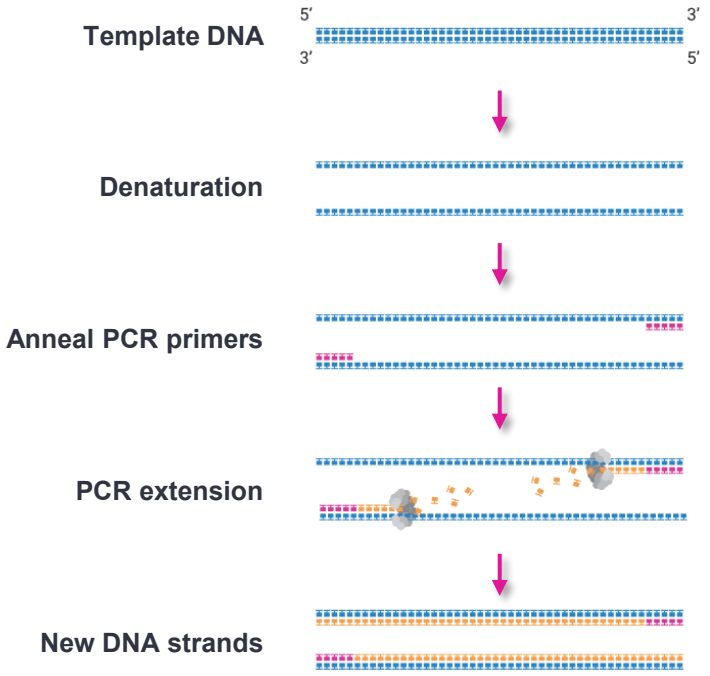
Consolidate legacy molecular workflows into one assay

Summary overview of PacBio targeted HiFi sequencing methods

Amplicon- and hybrid capture-based HiFi targeted sequencing workflows

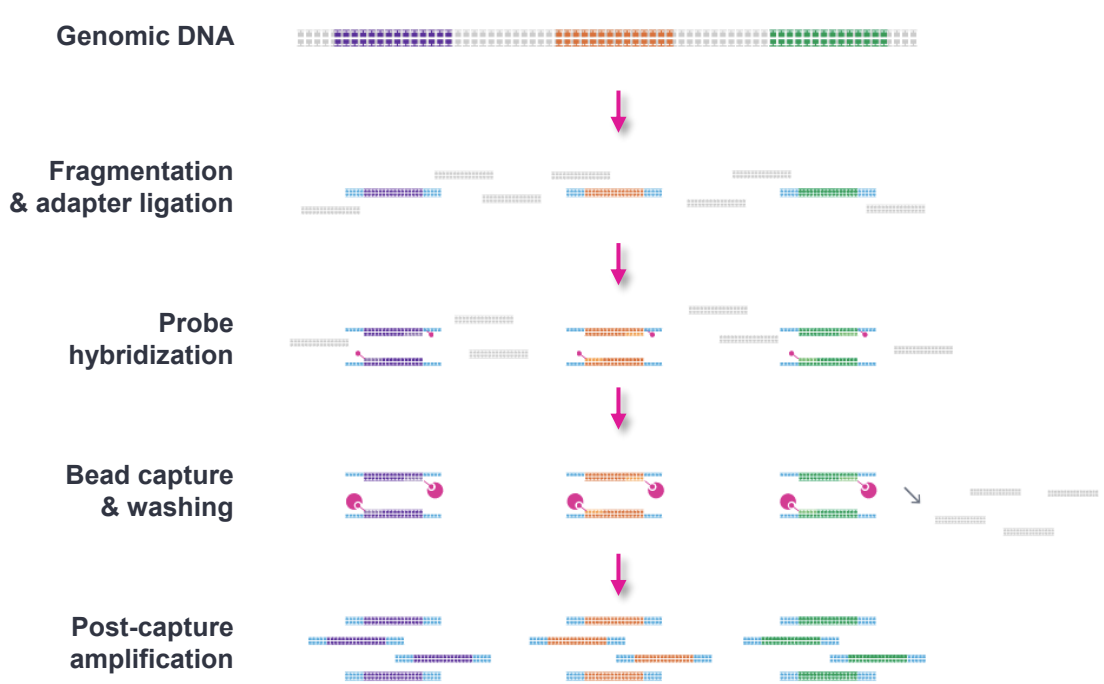
HiFi amplicon sequencing[1]

- Full-length sequencing of PCR amplicons
- Analysis of single genes or small panels
- Very low cost
- Supported indexing and library prep products



HiFi hybrid capture target enrichment sequencing[2]

- Hybridization capture with nucleic acid probes
- Analysis of small to large panels
- Low cost, high throughput, standard workflows
- Supported products from PacBio + PacBio Compatible partners



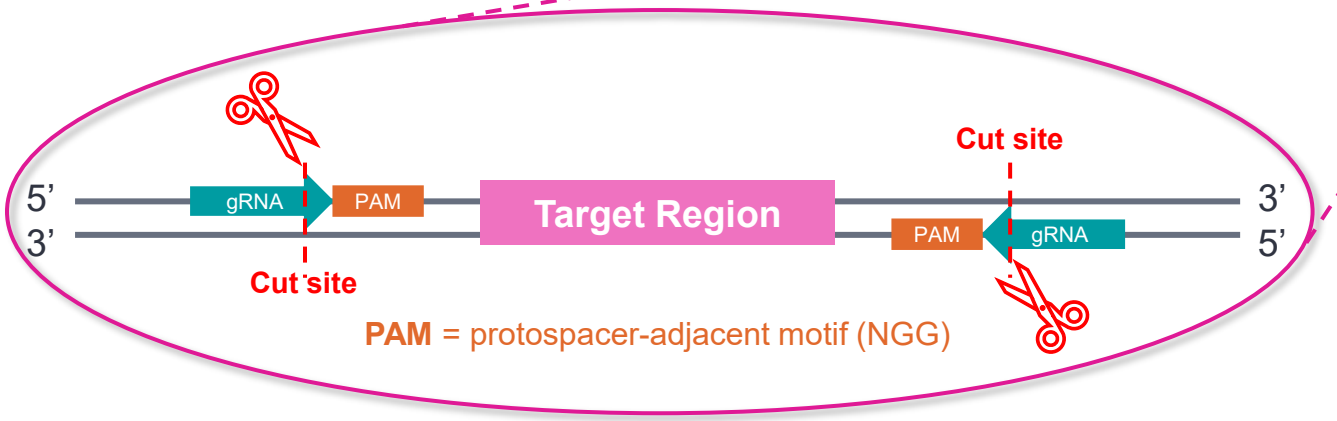
[1] See the following Application notes: ***HiFi amplicon sequencing for pharmacogenetics: CYP2D6*** (102-326-548), ***HiFi amplicon sequencing for congenital adrenal hyperplasia*** (102-326-547), and ***HiFi targeted sequencing and comprehensive analysis of SMN1/2 with Paraphase*** (102-326-566).
[2] See ***Application brief – HiFi target enrichment with Twist probes*** (102-326-515).

Summary overview of PacBio targeted HiFi sequencing methods (cont.)

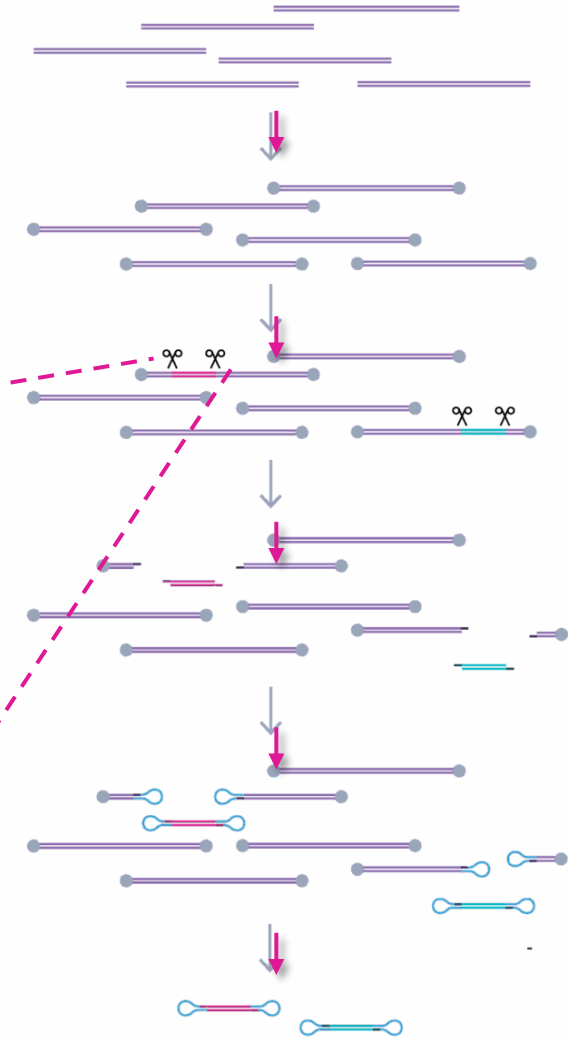
Amplification-free HiFi targeted sequencing workflow using PureTarget method

PureTarget sequencing method[1]

- Combines CRISPR-Cas9 technology for target enrichment and long and accurate HiFi reads to sequence the most challenging genomic regions at scale
- PureTarget libraries consist of DNA in its native form, including epigenetic marks like methylation and are free from errors and artifacts that can be introduced by PCR or hybridization capture methods
- Off-the-shelf panels of targets available for repeat expansion neurological disease and carrier screening research



4 – 5 kb[2]



Repair gDNA and dephosphorylate to block DNA ends

DNA digestion with Cas9 and guide RNAs

dA tailing of cut ends

Ligation of indexed SMRTbell adapters

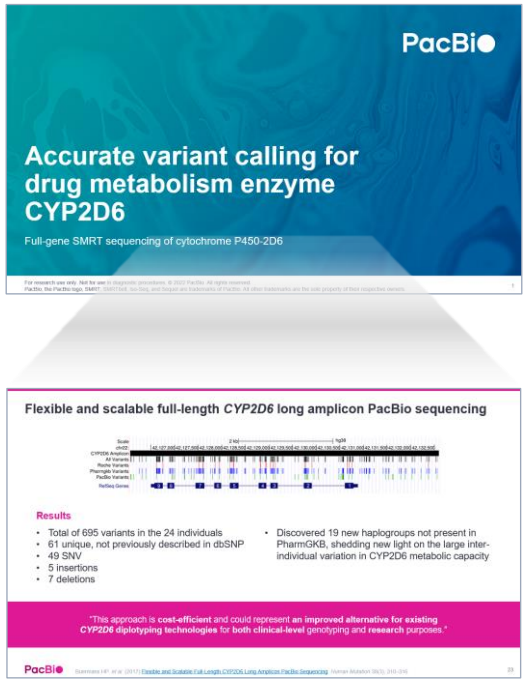
Nuclease digestion of non-SMRTbell templates and PureTarget library cleanup

[1] For information about performing targeted HiFi sequencing using the amplification-free **PureTarget** method, see ***PureTarget brochure – Shine a light on dark regions of the genome*** (102-326-609).

Multiplexed amplicon library preparation workflow overview

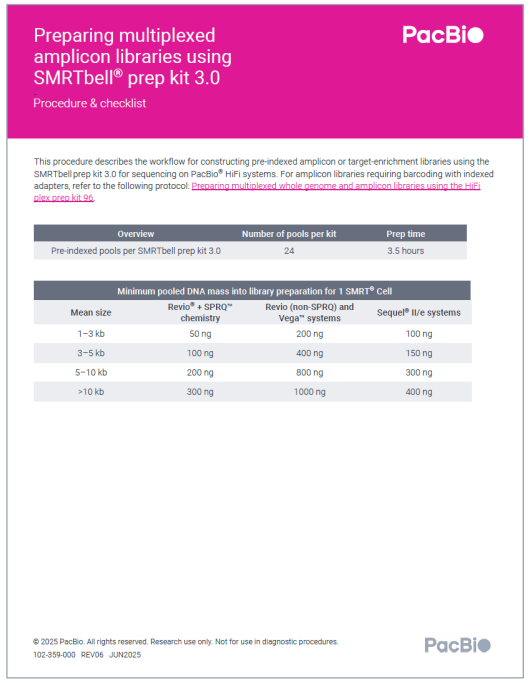
Multiplexed amplicon sequencing: How to get started

Application-specific educational literature → **Application-specific Procedure & checklist** → **Application-specific technical overviews** → **Library construction, sequencing & analysis**



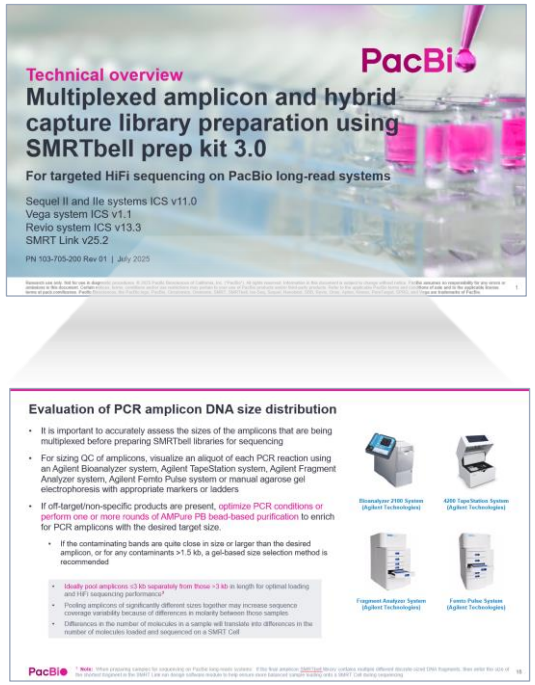
PacBio literature website (Link)

Application-specific brochures, informational guides and and other product literature containing best practices recommendations for sample preparation and data analysis workflows.



Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)

Technical documentation containing library construction and sequencing preparation protocol details



Technical overview: Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0 (103-705-200)

Technical overview presentations describe the end-to-end library preparation, sequencing setup and data analysis workflow for specific applications. Example sequencing performance data are also summarized.

PCR amplification

Use indexed PCR primers for barcoding
Use 50 – 1000 ng of pooled barcoded amplicon input for library construction

Library construction
(SMRTbell prep kit 3.0)

Construct pre-indexed amplicon or target-enrichment libraries using SMRTbell prep kit 3.0 (SPK 3.0)

HiFi sequencing
(Sequel IIe / Vega / Revio systems)

Perform ABC[1] and sequence libraries on PacBio long-read systems

Data analysis
(SMRT Link or third-party tools)

E.g., HiFi mapping, target enrichment analysis

[1] ABC = Anneal primer / Bind polymerase / Clean up bound complex

Indexing options for multiplexed amplicon HiFi sequencing[1]

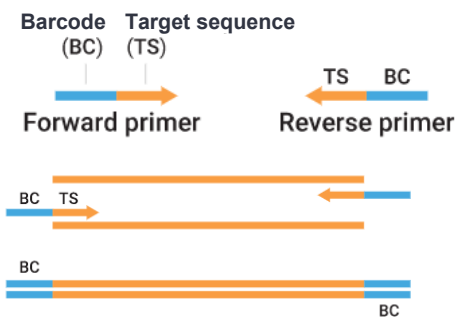
Indexed PCR primers

Use customer-supplied indexed PCR primers

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)

- Target-specific primers tailed with PacBio barcodes are used to produce symmetrically or asymmetrically barcoded samples with a 1-step PCR method.
- Barcoded amplicons are pooled into a single tube for SMRTbell library construction
- Can be used for small or large projects when validation of barcoded target-specific primers will be performed at the start of a project

1-Step PCR



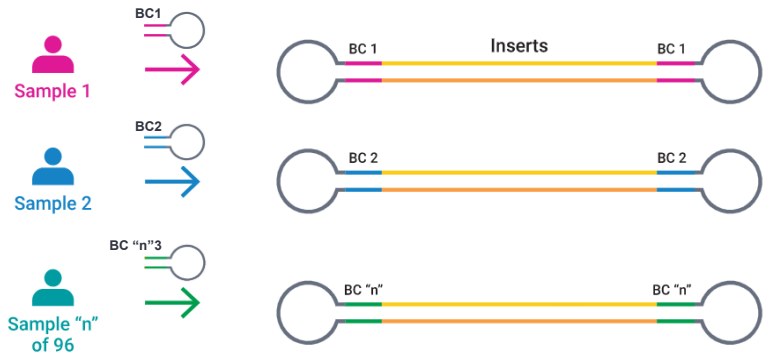
SMRTbell indexed adapters

Use PacBio SMRTbell indexed adapter plate (102-009-200)

Procedure & checklist — Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 (103-418-800)

- PacBio barcodes are added to amplicons through ligation of barcoded adapters during SMRTbell library construction to produce symmetrically barcoded samples.
- Barcoded amplicons are pooled into a single tube for subsequent sequencing on a single SMRT Cell
- Recommended for smaller projects (≤96 samples) using validated PCR systems and off-the-shelf assays.

SMRTbell indexed adapter ligation



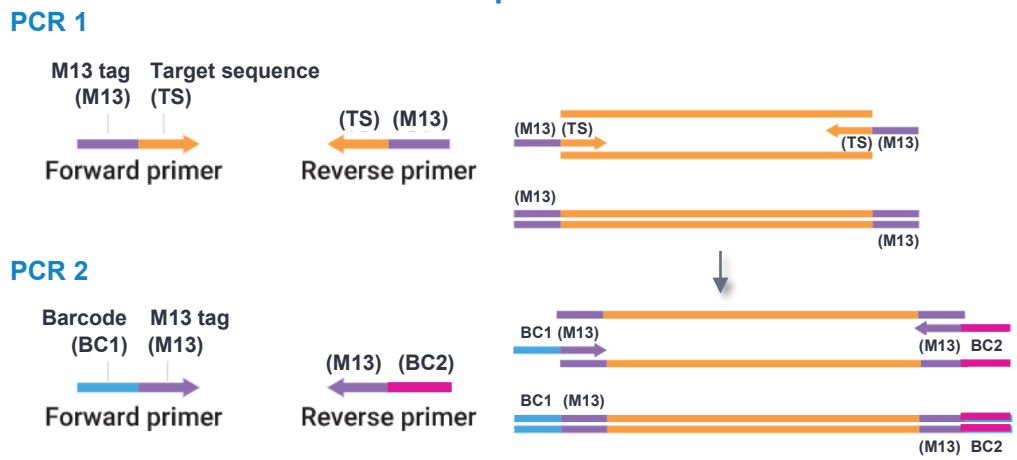
Indexed M13 primers

Use PacBio barcoded M13 primer plate (102-135-500)

Procedure & checklist – Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers and SMRTbell prep kit 3.0 (101-921-300)

- PacBio barcodes are added to amplicons through a 2-step PCR method using M13-tagged target-specific primers and M13-tagged barcoded primers to produce asymmetrically barcoded samples
- Barcoded amplicons are pooled into a single tube for SMRTbell library construction
- Recommended for larger projects (up to 384 samples)

2-Step PCR



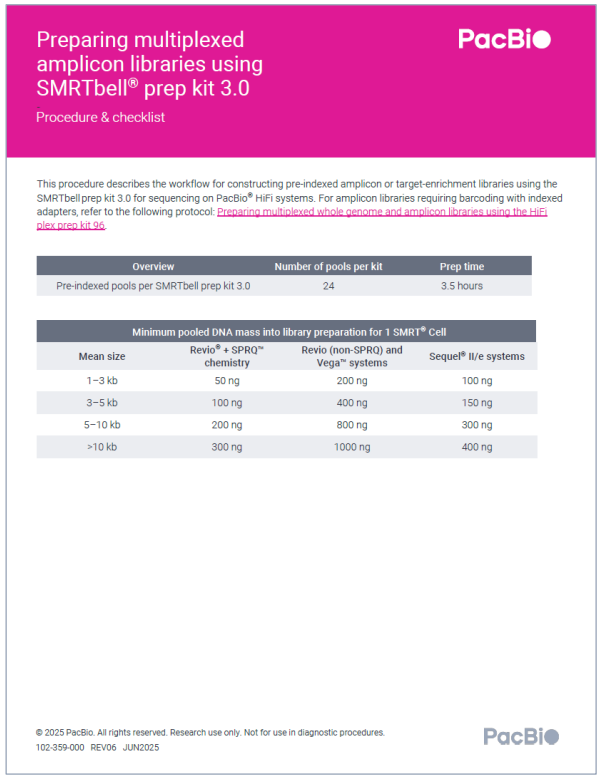
[1] Refer to PacBio's Multiplexing website for further details about multiplexing options. For information about multiplexing consumables, see the Multiplexing Kits website

SMRTbell prep kit 3.0 amplicon library preparation procedure description

Procedure & checklist – Procedure & checklist — Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000) describes the workflow for constructing pre-indexed amplicon[1] or target-enrichment libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio HiFi systems.

Overview	Number of pools per kit	Prep time
Pre-indexed pools per SMRTbell prep kit 3.0	24	3.5 hours

Minimum pooled DNA mass into library preparation for 1 SMRT® Cell			
Mean size	Revio® + SPRQ™ chemistry	Revio (non-SPRQ) and Vega™ systems	Sequel® II/e systems
1–3 kb	50 ng	200 ng	100 ng
3–5 kb	100 ng	400 ng	150 ng
5–10 kb	200 ng	800 ng	300 ng
>10 kb	300 ng	1000 ng	400 ng


Preparing multiplexed amplicon libraries using SMRTbell® prep kit 3.0

Procedure & checklist

PacBio

This procedure describes the workflow for constructing pre-indexed amplicon or target-enrichment libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio® HiFi systems. For amplicon libraries requiring barcoding with indexed adapters, refer to the following protocol: Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96.

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102-359-000 REV06 JUN2025

PacBio

PacBio Documentation (102-359-000)

Note: For amplicon libraries requiring barcoding with indexed adapters, refer to the following protocol: ***Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800).

[1] **Note:** For amplicon libraries requiring barcoding with indexed adapters, refer to the following protocol: ***Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800).

SMRTbell prep kit 3.0 amplicon library preparation workflow overview

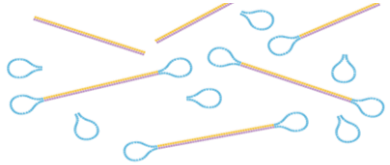
SPK 3.0 library preparation with pre-indexed input amplicon DNA samples can be completed in ~3.5 hours[1]

Amplicon library preparation workflow

Input (pre-indexed) amplicon DNA QC & cleanup

DNA repair & A-tailing

Adapter ligation & cleanup



Nuclease treatment & cleanup

Protocol documentation or reference

SPK 3.0 amplicon protocol reference[1]

Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000)

Recommended equipment & consumables

SMRTbell prep kit 3.0

[1] **Note:** For amplicon libraries requiring barcoding with indexed SMRTbell adapters, refer to the following protocol: ***Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800).

SMRTbell prep kit 3.0 bundle (102-182-700)

SPK 3.0 bundle supports amplicon library preparation workflows[1]

- Contains the necessary reagents for library preparation with SMRTbell adapters
- Kit also includes SMRTbell cleanup beads and low TE buffer
- Supports 24 SMRTbell libraries per kit
- Compatible with sequencing on the Sequel IIe system, Vega system and Revio system

SMRTbell prep kit 3.0 bundle components

Component		Description
1		**SMRTbell prep kit 3.0** • Contains core reagents for SMRTbell template construction
2		**Low TE buffer** • For DNA shearing and cleanup
3		**SMRTbell cleanup beads** • For DNA cleanup

SMRTbell prep kit 3.0 bundle configuration

SMRTbell prep kit 3.0 (102-141-700)

#	Component	Part number	Qty	Color	Volume
1	Repair buffer	102-166-000	1	purple	220 µL
2	End repair mix	102-166-100	1	blue	110 µL
3	DNA repair mix	102-167-700	1	green	55 µL
4	SMRTbell adapter	102-167-800	1	orange	125 µL
5	Ligation mix	102-167-200	1	yellow	860 µL
6	Ligation enhancer	102-179-100	1	red	55 µL
7	Nuclease buffer	102-167-900	1	light purple	155 µL
8	Nuclease mix	102-166-200	1	light green	155 µL
9-10	Elution buffer	100-159-800	2	white	1.5 mL

Low TE buffer (102-178-400)

#	Component	Part number	Qty	Color	Volume
1	Low TE buffer (pH 8.0)	102-178-400	1	clear	10 mL

SMRTbell cleanup beads (102-158-300)

#	Component	Part number	Qty	Color	Volume
1	SMRTbell cleanup beads	102-158-300	1	clear	10 mL

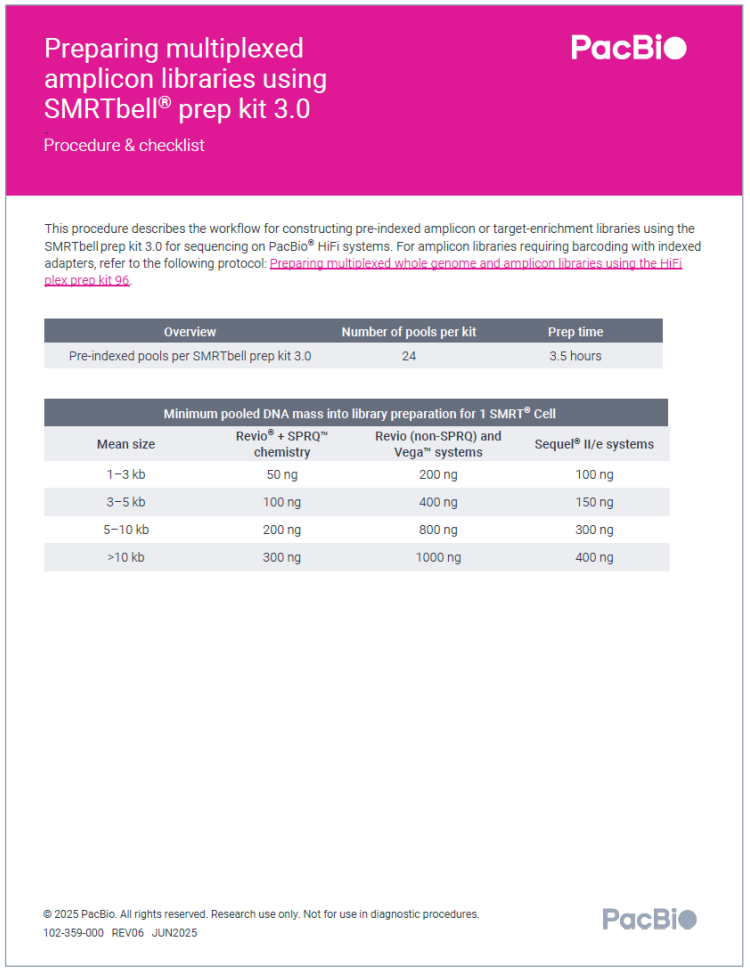
Multiplexed amplicon library preparation workflow details

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000)

Procedure & checklist 102-359-000 describes the workflow for constructing pre-indexed amplicon or target-enrichment libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio HiFi systems.

Procedure & checklist contents

1. Amplicon DNA input QC recommendations and general best practices for reagent & sample handling.
2. Enzymatic workflow steps for SMRTbell library construction using SMRTbell prep kit 3.0.
3. Workflow steps for sample setup ABC[1] (annealing, binding, and cleanup) to prepare samples for sequencing using Vega polymerase kit or Revio SPRQ polymerase kit.
4. Additional information on DNA shearing and multiplexing guidance for hybrid capture samples.



Preparing multiplexed amplicon libraries using SMRTbell® prep kit 3.0

Procedure & checklist

PacBio

This procedure describes the workflow for constructing pre-indexed amplicon or target-enrichment libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio® HiFi systems. For amplicon libraries requiring barcoding with indexed adapters, refer to the following protocol: Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96.

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102-359-000 REV06 JUN2025

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PacBio Documentation (102-359-000)

[1] For primer annealing, polymerase binding & complex cleanup (ABC) using Vega chemistry or Revio SPRQ chemistry, follow sample setup instructions in Procedure & checklist – **Do not use** SMRT Link Sample Setup. For ABC using Sequel IIe system chemistry or Revio non-SPRQ chemistry, follow SMRT Link Sample Setup ABC Calculator software for preparing samples for sequencing.

Key amplicon library preparation and sequencing workflow steps

Amplicon library preparation + ABC workflow can be completed within 1 day[1]

QC

1 Input amplicon DNA QC & cleanup

Qubit 4 fluorometer system (ThermoFisher Sci.)
DNA sizing QC tool (TapeStation system or other tool)

Refer to third-party user guide documenation

Safe stop

Library construction

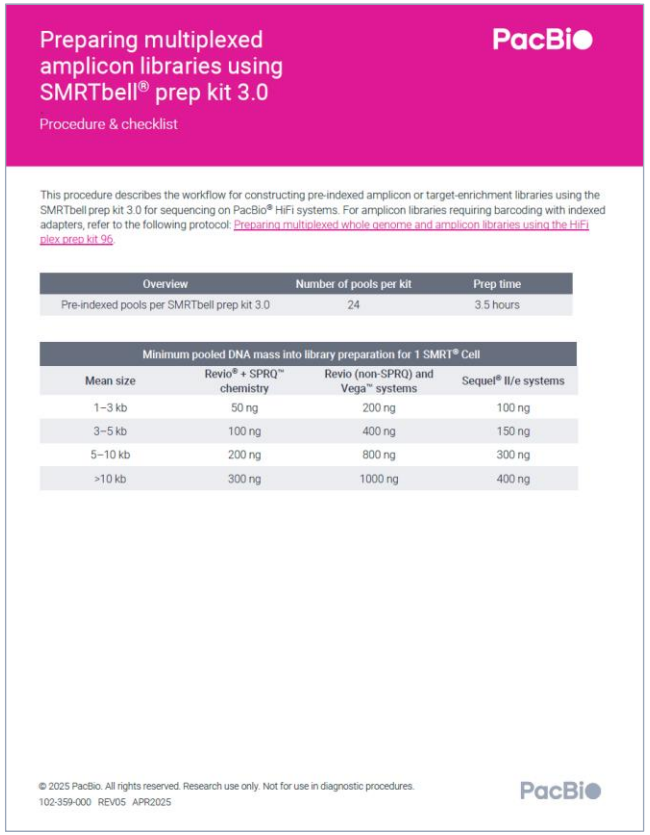
2 Repair & A-tailing	45 min	
3 Adapter ligation & cleanup	45 min	 SMRTbell prep kit 3.0 (102-182-700) (for SMRTbell library construction)
Safe stop		
4 Nuclease treatment & cleanup	30 min	

ABC

5 Annealing, binding & cleanup (ABC)	1 hr	 Revio SPRQ polymerase kit (103-496-900) *or* Revio polymerase kit (non-SPRQ) (102-739-100) *or* Vega polymerase kit (103-426-500)

Amplicon protocol reference

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0
(102-359-000)



Preparing multiplexed amplicon libraries using SMRTbell® prep kit 3.0

PacBio

Procedure & checklist

This procedure describes the workflow for constructing pre-indexed amplicon or target-enrichment libraries using the SMRTbell prep kit 3.0 for sequencing on PacBio® HiFi systems. For amplicon libraries requiring barcoding with indexed adapters, refer to the following protocol: Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96.

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102-359-000 REV05 APR2025

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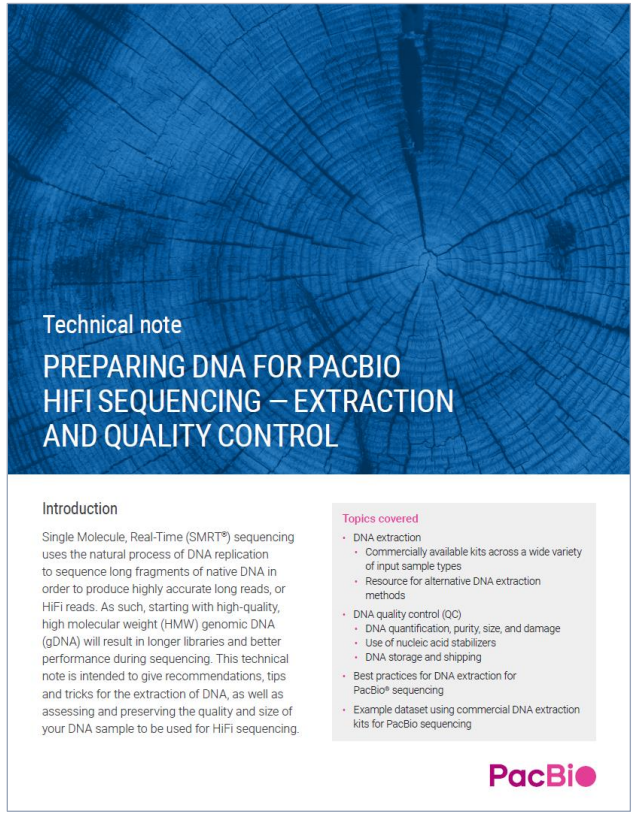
[1] Example workflow step times shown are for manually processing up to 8 pre-indexed amplicon samples.

Best practices for generating high-quality PCR amplicons

- Clean, target-specific PCR products are extremely important for obtaining high-quality sequence data
- Non-specific products can represent a substantial percentage of the sequencing reads if they are not removed
- To minimize their presence, consider the recommendations described below for generating high-quality amplicons suitable for SMRTbell library preparation and sequencing

1. Begin with high-quality nucleic acids and work in a clean environment.
    - For targeted sequencing of genomic DNA (gDNA) samples, starting with high-quality DNA will result in better performance during sequencing.
        - PacBio Technical Note: DNA Prep (102-193-651) provides recommendations, tips and tricks for genomic DNA extraction as well as assessing and preserving the quality and size of your DNA sample to be used for PacBio HiFi sequencing
        - PacBio Technical Note: Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500) outlines best practices for extracting human whole blood samples using Nanobind kits
    - If extracted nucleic acids must be stored, freeze at high concentrations in appropriately-buffered solutions.
    - To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, sub-aliquot DNA into smaller volumes for storage.
    - Set up PCR reactions in an environment free from sources of non-specific primer and template contaminants, ideally a laminar flow hood, using dedicated pre-PCR pipettor, tips and reagents.



PacBio Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)

Best practices for generating high-quality PCR amplicons (cont.)

2. Use PCR reagents and conditions for generating target-specific, full-length amplicons.
    - Use the highest-fidelity polymerase compatible with your PCR amplification system.
    - Use desalted or HPLC-purified oligo primers; damaged bases at the ends of the amplicons cannot be repaired by DNA Damage Repair enzymes.
    - Optimize PCR conditions to minimize total time spent at high (>65°C) temperatures, particularly during denaturation.
    - PCR extension time should be long enough to ensure complete extension, taking into consideration the polymerase used and target amplicon size.
        - For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps.
        - As a general guideline, use extension times of one minute per 1000 base pairs (e.g., 3 minutes for a 3 kb product).
3. Use the lowest number of cycles required for obtaining adequate yields (ng) of PCR products to proceed with SMRTbell library construction. Avoid over-amplification.
4. If non-specific products are present, optimize PCR conditions or perform AMPure PB bead-based size selection to enrich for PCR amplicons with the desired target size
5. Note: Using gel-extracted amplicon products may result in lower sequencing performance due to the damage inherently caused by intercalating dyes such as ethidium bromide and exposure to UV radiation.
    - Sequencing amplicons stained with SYBR dyes from ThermoFisher Scientific is untested, and thus, also **not** recommended.
    - If working with a gel product that has been stained with a dye, it is recommended to bring it through **additional rounds of amplification** to remove damage and dyes prior to library prep and sequencing.

Evaluation of PCR amplicon DNA concentration

- For accurate quantification of PCR samples to be used in multiplexed amplicon library preparation workflows, PacBio recommends using the Qubit fluorometer and Qubit dsDNA High Sensitivity (HS) assay reagents (Thermo Fisher Scientific)
  - Qubit dsDNA HS assay quantitation range: 0.2 – 100 ng
  - **Note:** When measuring very low DNA concentrations of amplicon samples (especially <1 kb library insert size), it may be helpful to **increase the sample aliquot volume above 1 µL** (up to 20 µL) in order to ensure sufficient assay sensitivity

Qubit dsDNA HS Assay Kit

Qubit 4 Fluorometer

Evaluation of PCR amplicon DNA purity

- DNA purity can be determined by using a NanoDrop system [Thermo Fisher Scientific] or other spectrophotometer tool
- For ultrapure DNA, A260/280 ratio is typically between ~1.8 - 2.0 and A260/230 ratio is ≥2.0
- **Note:** High UV absorbance values are *not* always a guarantee of optimal sequencing performance because not all inhibitors absorb at the wavelengths of 230, 260, and 280 nm.
- Conversely, low UV absorbance values are *not* always a guarantee that non-optimal sequencing performance will be obtained for a sample

A260/A280 Ratio

- A low A260/A280 ratio may be the result of:
  - Protein
  - Phenol
  - Other contaminants that absorb strongly at or near 280 nm
  - Sometimes it may be caused by a very low concentration of nucleic acid.
- High 260/280 ratios are not indicative of an issue

If A260/280 and A260/230 readings are out of the recommended ranges, perform one or more rounds of **bead purification (e.g., SMRTbell cleanup beads, AMPure PB beads)** followed by re-assessment of the quantity and purity of the input DNA sample.[1]

A260/A230 Ratio

- A low A260/A230 ratio may be the result of:
  - Protein[2]
  - Carbohydrate carryover (often a problem with plants)
  - Residual phenol from nucleic acid extraction
  - Residual guanidine (often used in column-based kits)
  - Glycogen used for precipitation
- A high A260/A230 ratio may be the result of:
  - Making a blank measurement on a dirty pedestal of a Nanodrop instrument
  - Using an inappropriate solution for the blank measurement

PacBio

1 See PacBio Technical Note: Preparing DNA for PacBio HiFi sequencing - Extraction and quality control (102-193-651)
2 See NEB Technical Note: A Practical Guide to Analyzing Nucleic Acid Concentration and Purity with Microvolume Spectrophotometers (2019)

Evaluation of PCR amplicon DNA size distribution

- It is important to accurately assess the sizes of the amplicons that are being multiplexed before preparing SMRTbell libraries for sequencing
- For sizing QC of amplicons, visualize an aliquot of each PCR reaction using an Agilent Bioanalyzer system, Agilent TapeStation system, Agilent Fragment Analyzer system, Agilent Femto Pulse system or manual agarose gel electrophoresis with appropriate markers or ladders
- If off-target/non-specific products are present, optimize PCR conditions or perform one or more rounds of AMPure PB bead-based purification to enrich for PCR amplicons with the desired target size.
    - If the contaminating bands are quite close in size or larger than the desired amplicon, or for any contaminants >1.5 kb, a gel-based size selection method is recommended

- Ideally pool amplicons ≤3 kb separately from those >3 kb in length for optimal loading and HiFi sequencing performance[1]
- Pooling amplicons of significantly different sizes together may increase sequence coverage variability because of differences in molarity between those samples
- Differences in the number of molecules in a sample will translate into differences in the number of molecules loaded and sequenced on a SMRT Cell

Bioanalyzer 2100 System (Agilent Technologies)

4200 TapeStation System (Agilent Technologies)

Fragment Analyzer System (Agilent Technologies)

Femto Pulse System (Agilent Technologies)

[1] **Note:** When preparing samples for sequencing on PacBio long-reads systems: If the final amplicon SMRTbell library contains multiple different discrete-sized DNA fragments, then enter the size of the shortest fragment in the SMRT Link run design software module to help ensure more balanced sample loading onto a SMRT Cell during sequencing.

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Recommended DNA input amount and quality

Indexed amplicon DNA input amount required for SMRTbell library preparation using SMRTbell prep kit 3.0

- The total amount of DNA required for constructing a SMRTbell library is dependent on the mean size of the amplicons being sequenced
- **Indexed amplicon DNA samples** can be **pooled** prior to library prep to satisfy the DNA input mass requirements (see Table below)
- The pooled amplicon mass required **per sample** can be calculated by dividing the total mass required by the number of samples per indexed pool
- Refer to table below for the minimum required total input DNA mass into library preparation per SMRT Cell for different insert sizes
- It is recommended to **pool amplicons by the bins specified in the Table below to achieve optimal loading** on PacBio long-read 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**

Recommended pooled amplicon DNA input mass, binned by size, into SMRTbell library preparation for different PacBio long-read sequencing platforms.

Minimum pooled amplicon DNA mass into SMRTbell library preparation for 1 SMRT Cell			
Mean amplicon DNA size	**Sequel IIe system chemistry**	**Revio (non-SPRQ) chemistry and Vega system chemistry**	**Revio system with SPRQ chemistry**
1 – 3 kb	100 ng per SMRT Cell	200 ng per SMRT Cell	50 ng per SMRT Cell
3 – 5 kb	150 ng per SMRT Cell	400 ng per SMRT Cell	100 ng per SMRT Cell
5 – 10 kb	300 ng per SMRT Cell	800 ng per SMRT Cell	200 ng per SMRT Cell
>10 kb	400 ng per SMRT Cell	1000 ng per SMRT Cell	300 ng per SMRT Cell

[1] **Note:** When preparing samples for sequencing on PacBio long-reads systems: If the final amplicon SMRTbell library contains multiple different discrete-sized DNA fragments, then enter the size of the shortest fragment in the SMRT Link run design software module to help ensure more balanced sample loading onto a SMRT Cell during sequencing.

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Amplicon library construction yields

Expected SPK 3.0 amplicon library construction yield

- Overall SMRTbell library construction yield is dependent on input amplicon DNA quality and size
  - The recovery from input amplified DNA to polymerase-bound SMRTbell library typically ranges between ~20 – 40% (includes SMRTbell library construction and ABC)

Protocol step	Expected DNA or SMRTbell step recovery	Expected DNA or SMRTbell overall recovery	Example DNA or SMRTbell overall recovery
Starting amplified DNA input	100%	100%	200 ng
Post-SMRTbell adapter ligation & SMRTbell bead cleanup	80 – 95%	80 – 95%	160 – 190 ng
Post-nuclease (pre-cleanup)	40 – 50%	32 – 48%	64 – 95 ng
Post-1.3X SMRTbell bead cleanup	75 – 80%	24 – 38%	48 – 76 ng
Post-ABC cleanup	75 – 95%	18 – 36%	36 – 72 ng

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Amplicon library construction yields (cont.)

Minimum polymerase-bound pooled amplicon library mass needed to load a SMRT Cell

- Minimum polymerase-bound pooled amplicon library mass needed to load a SMRT Cell is dependent on amplicon library insert size and desired on-plate loading concentration (OPLC)[1]

Minimum polymerase-bound pooled amplicon library mass needed to load a SMRT Cell for different PacBio long-read sequencing platforms.

Polymerase-bound pooled amplicon library mass needed to load one SMRT Cell[1]			
Mean amplicon DNA size	**Sequel IIe system chemistry (150 OPLC)**	**Revio (non-SPRQ) chemistry and Vega system chemistry (300 pM OPLC)**	**Revio system with SPRQ chemistry (300 pM OPLC)**
1 – 3 kb	35 ng	60 ng	15 ng
3 – 5 kb	60 ng	100 ng	25 ng
5 – 10 kb	120 ng	200 ng	50 ng
>10 kb	175 ng	300 ng	75 ng

[1] Refer to appropriate *Procedure and checklist* documentation for the most up-to-date guidance on recommended sample on-plate loading concentration (OPLC) range to use for sequencing amplicon libraries using different PacBio long-read systems.

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling – PacBio kits or consumables[1]

PacBio kit or consumable	Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice	Bring these reagents to room temperature 30 minutes prior to use
SMRTbell prep kit 3.0 (102-182-700) 	❑ Repair buffer	 ❑ End repair mix	❑ Elution buffer
	❑ Nuclease buffer	 ❑ DNA repair mix	❑ SMRTbell cleanup beads
	❑ SMRTbell adapter	 ❑ Ligation mix	
	❑ Elution buffer	 ❑ Ligation enhancer	
		 ❑ Nuclease mix	
		 ❑ SMRTbell adapter	

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling – PacBio kits or consumables[1] (cont.)

PacBio reagent handling notes

- Room temperature is defined as any temperature in the range of 18 – 23°C for this protocol
- Once thawed, reaction buffers may be stored on cold block, at 4°C, or on-ice prior to making master mix
- Mix reagent buffers with a brief vortex prior to use (do not vortex enzymes)
- Briefly spin down all reagent tubes in a microcentrifuge to collect all liquid at the bottom
- Vortex SMRTbell cleanup beads immediately before use (failure to do this will result in low recovery)
- Pipette-mix (e.g., up and down 10 times) all bead binding and elution steps until beads are distributed evenly in solution
- Samples can be stored at 4°C at all safe stopping points listed in the protocol

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

General best practices recommendations for preparing amplicon libraries using SMRTbell prep kit 3.0 (cont.)

Sequencing preparation (ABC) and polymerase-bound library storage

- ***Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0*** (102-359-000) includes instructions for the primer annealing, polymerase binding & complex cleanup (ABC) sample setup steps for Revio and Vega systems
  - → For sequencing amplicon SMRTbell libraries on the Revio system with SPRQ chemistry or the Vega system: Follow sample setup instructions stated in the protocol to perform ABC and final loading dilution procedure – Do not use SMRT Link Sample Setup software
- Sequencing polymerase is stable once bound to the SMRTbell library and can be stored at 4°C or frozen at -20°C.
- Stored polymerase-bound library shows equivalent loading to freshly prepared bound libraries up to the recommendations listed below.

  Recommended polymerase-bound storage:[1]
  - Polymerase-bound libraries can be stored at 4°C for up to 1 month
  - Polymerase-bound libraries can be stored at -20°C for at least 6 months
  - Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles
- Stored polymerase-bound libraries should be protected from light since Loading buffer is light-sensitive

[1] Polymerase-bound storage recommendations described here apply to SMRTbell libraries bound to either Sequel II polymerase, Vega polymerase, Revio polymerase or Revio SPRQ polymerase.

Amplicon DNA sample QC

Perform input amplicon DNA QC using a Qubit dsDNA HS assay and a DNA sizing tool

1 Input amplicon DNA QC & cleanup

↓ Safe stop

2 Repair & A-tailing

↓

3 Adapter ligation & cleanup

↓ Safe stop

4 Nuclease treatment & cleanup

↓

5 Annealing, binding & cleanup (ABC)

DNA quantification QC

Qubit 4 fluorometer (Thermo Fisher Scientific)

Qubit fluorometer in conjunction with Qubit 1X dsDNA high-sensitivity assay (Thermo Fisher Scientific) enables rapid, specific and accurate determination of nucleic acid concentrations in a single sample[1,2]

- Assay is highly selective for dsDNA over ssDNA, RNA, protein, and free nucleotides. Contaminants, such as salts, solvents, or detergents are well-tolerated.
- Depending on sample volume, assay kit is designed to be accurate for initial DNA sample concentrations of 5 pg/µL to 120 ng/µL, providing a detection range of 0.1−120 ng.

DNA sizing QC

Bioanalyzer 2100 System (Agilent Technologies)

4200 TapeStation System (Agilent Technologies)

Fragment Analyzer System (Agilent Technologies)

Femto Pulse System (Agilent Technologies)

Measure the DNA size distribution of the pool with the appropriate sizing technology following the manufacturer's instructions

- Amplicons ≤10 kb: Agilent 2100 Bioanalyzer, TapeStation, or Fragment Analyzer
- Amplicons ≥10 kb: Agilent Femto Pulse system

[1] **Important: The Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer (concentration readings will not be accurate).**.

[2] **Note:** We do not recommend quantification with UV-Vis Spectrophotometers (e.g., NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples.

DNA repair & A-tailing

Repair sites of DNA damage and prepare amplicon DNA for ligation to SMRTbell adapter

1 Input amplicon DNA QC & cleanup

↓ Safe stop

2 Repair & A-tailing

↓

3 Adapter ligation & cleanup

↓ Safe stop

4 Nuclease treatment & cleanup

↓

5 Annealing, binding & cleanup (ABC)

2. DNA repair & A-tailing

✔	Step	Instructions
	2.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip Repair master mix steps (Steps 2.2 to 2.4).

Repair master mix

✔	Tube	Component	Volume per pool
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	14 µL

2.2	Pipette-mix **Repair master mix**.
2.3	Quick-spin **Repair master mix** in a microcentrifuge to collect liquid.
2.4	Add **14 µL** of the **Repair master mix** to each sample. The total reaction volume should be **60 µL**.
2.5	Pipette-mix each sample.
2.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.
2.7	Run the repair & A-tailing thermocycler program.

Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

2.8	Proceed to the next step of the protocol.

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of sample pools being prepared (e.g., 4, 8, 16 or 24[1]), plus 10% overage

- Run **Repair and A-tailing** thermal cycler program
- Set the lid temperature to 75°C if programmable
  - If the lid temperature is not programmable, it is acceptable to leave at 95–105°C

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Adapter ligation & cleanup

Ligate SMRTbell adapter to the ends of each DNA fragment

1. Input amplicon DNA QC & cleanup
2. Safe stop
3. Repair & A-tailing
4. Safe stop
5. **Adapter ligation & cleanup**
6. Nuclease treatment & cleanup
7. Annealing, binding & cleanup (ABC)

3. Adapter ligation & cleanup

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
	3.1	Add the following components in the order and volume listed below to a microfuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip **Ligation master mix** steps (steps 3.3 to 3.5).

Ligation master mix

✔	Tube	Component	Volume per pool
	Orange	SMRTbell adapter	4 µL
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	35 µL

	3.2	Pipette-mix **Ligation master mix**.
	3.4	Quick-spin **Ligation master mix** in a microcentrifuge to collect liquid.
	3.5	Add **35 µL** of **Ligation master mix** to each sample from previous step. The total volume should be **95 µL**.
	3.8	Run the adapter ligation thermocycler program.

Step	Time	Temperature
1	30 min	20°C
2	Hold	4°C

Cleanup with 1.3X SMRTbell cleanup beads

	3.9	Add **124 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	3.10	Pipette-mix the beads until evenly distributed.
	3.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	3.12	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	3.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of sample pools being prepared (e.g., 4, 8, 16 or 24[1]), plus 10% overage

- Run **Adapter ligation** thermal cycler program
- Set the lid temperature to 75°C if programmable
  - If the lid temperature is not programmable, it is acceptable to leave at 95–105°C

- Perform **1.3X SMRTbell bead cleanup**
- ***IMPORTANT!*** Allow SMRTbell cleanup beads to come up to room temperature by bringing them out of 4°C storage at least 30 min prior to use

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Nuclease treatment & cleanup

Remove unligated DNA fragments and leftover SMRTbell adapters from the sample

1. Input amplicon DNA QC & cleanup
2. Repair & A-tailing
3. Adapter ligation & cleanup
4. **Nuclease treatment & cleanup**
5. Annealing, binding & cleanup (ABC)

Safe stop

Safe stop

4. Nuclease treatment

✔	Step	Instructions
	4.1	Add the following components in the order and volume listed below to a microfuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip **Nuclease master mix** steps (steps 4.2 to 4.4).

Nuclease master mix

✔	Tube	Component	Volume per pool
	Light purple	Nuclease buffer	5 µL
	Light green	Nuclease mix	5 µL
		Total volume	10 µL

	4.2	Pipette-mix **Nuclease master mix**.
	4.3	Quick-spin **Nuclease master mix** in a microcentrifuge to collect liquid.
	4.4	Add **10 µL** of **Nuclease master mix** to each sample. Total volume should equal **50 µL**.
	4.5	Pipette-mix each sample.
	4.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	4.7	Run the nuclease treatment thermocycler program.

Step	Time	Temperature
1	15 min	37°C
2	Hold	4°C

- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube[1]
- Adjust component volumes for the number of sample pools being prepared (e.g., 4, 8, 16 or 24[1]), plus 10% overage

- Run **Nuclease treatment** thermal cycler program
- Set the lid temperature to 75°C if programmable
  - If the lid temperature is not programmable, it is acceptable to leave at 95–105°C

PacBio

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Nuclease treatment & cleanup

Perform final cleanup using 1.3X SMRTbell cleanup beads

1. Input amplicon DNA QC & cleanup
2. Repair & A-tailing
3. Adapter ligation & cleanup
4. Nuclease treatment & cleanup
5. Annealing, binding & cleanup (ABC)

Safe stop

Safe stop

4. Cleanup with 1.3X SMRTbell cleanup beads

✔	Step	Cleanup with 1.3X SMRTbell cleanup beads
	4.8	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	4.9	Pipette-mix the beads until evenly distributed.
	4.10	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	4.11	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	4.12	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.13	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	4.14	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.
	4.15	Repeat the previous step.
	4.17	Remove the tube strip from the magnetic rack. **Immediately** add **26 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	4.18	Quick-spin the tube strip in a microcentrifuge.
	4.19	Incubate at **room temperature** for **5 minutes** to elute DNA.
	4.20	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.21	Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a **new tu strip**. Discard old tube strip with beads.
	4.22	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure DNA concentration with a Qubit Fluorometer using the 1x dsDNA HS kit. Calculate the total mass.
	4.23	Proceed to Section 5 to prepare 25 µL of library for sequencing on the Vega system or Revio (+SPRQ). If necessary, **dilute 25 µL of SMRTbell library to the concentrations indicated below**. Failure to normalize libraries or pools of libraries to the appropriate concentration prior to ABC may result in low sequencing yield.

SMRTbell library size	Concentration (ng/µL)
<3 kb	<10 ng/µL
3–10 kb	<20 ng/µL
>10 kb	<60 ng/µL

- Perform **1.3X SMRTbell bead cleanup**
- ***IMPORTANT!*** Allow SMRTbell cleanup beads to come up to room temperature by bringing them out of 4°C storage at least 30 min prior to use

- After eluting in **26 µL** of EB, perform **DNA concentration QC** on final amplicon library using Qubit assay

- **Note:** Final amplicon library concentration must be **<10 ng/ µL** for <3 kb insert sizes, **<20 ng/ µL** for 3-10 kb insert sizes and **<60 ng/ µL** for inserts >10 kb to proceed with annealing, binding & cleanup (ABC)
  → Using a concentration higher than recommended for ABC may negatively impact sequencing performance

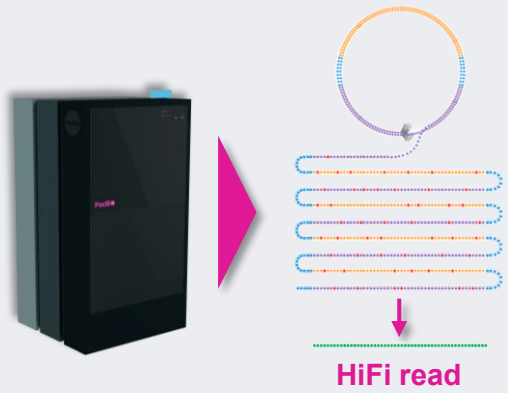
To prepare amplicon samples for sequencing using Revio SPRQ or Vega chemistry, follow ABC workflow instructions in **Step 5** using the recommended loading concentration[1]

[1] **Note:** To prepare amplicon samples for sequencing using Sequel IIe system chemistry or Revio non-SPRQ chemistry, follow ABC workflow instructions in SMRT Link Sample Setup software module.

Multiplexed amplicon library sequencing preparation workflow details

Sample Setup & Run Design recommendations for SPK 3.0 multiplexed amplicon libraries – Revio system

Workflow	Key setup parameters	Revio system recommended settings
		Amplicon samples (0.5 kb to >10 kb)
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Revio (non-SPRQ) polymerase kit / Revio SPRQ polymerase kit
	Concentration on plate (OPLC)	300 – 400 pM
Run design	Application type	<3 kb amplicons / >=3 kb amplicons
	Library type	Standard
	Insert size	Specify an insert size (500 base pairs minimum)[1]
	Movie acquisition time	12 hrs (<5 kb) / 24 hrs (~5 – 20 kb)
	Use adaptive loading	YES
	Data options[2]	Include base kinetics = NO Consensus Mode = MOLECULE[3]



Recommended target *P1* loading range for Revio system is **~50 – 70%**

[1] The insert size is the length of the double-stranded nucleic acid fragment in a SMRTbell template, excluding the hairpin adapters. This matches the average insert size for the sample; the size range boundaries are described in the library preparation protocol. **Note:** If the library contains a mixture of multiple discrete-sized fragments, then enter the size of the shortest fragment to help produce more balanced loading of different fragment sizes in the sample.

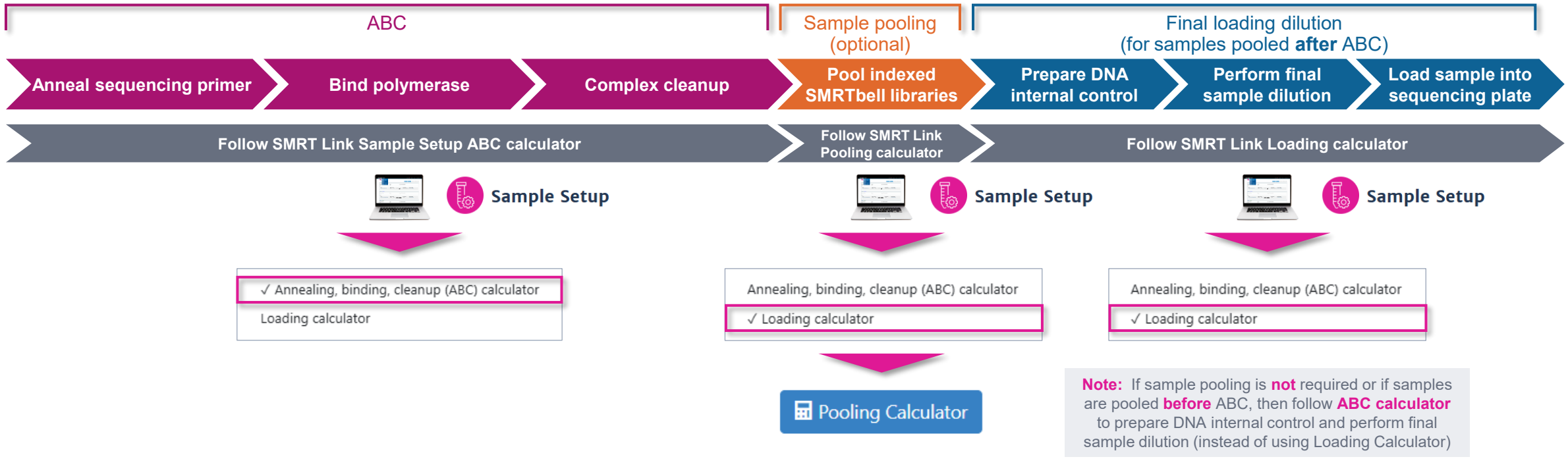
[2] For PCR-indexed amplicon libraries ligated to standard (non-barcoded) SMRTbell adapters, specify **Sample is indexed = NO** in the SMRT Link run design and then manually perform sample demultiplexing in SMRT Link using Demultiplex Barcodes data utility.

[3] **Note:** By default, Consensus mode is automatically set to **MOLECULE** for <3 kb amplicon samples and >=3 kb amplicon samples.

Sample setup workflow overview for Revio (non-SPRQ) polymerase libraries

For binding libraries with Revio (non-SPRQ) polymerase kit, follow SMRT Link Sample Setup ABC calculator instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 amplicon library	**Revio (non-SPRQ) polymerase kit** (102-817-600)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link Sample Setup ABC calculator
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator (if pooling samples **after** ABC)[1]



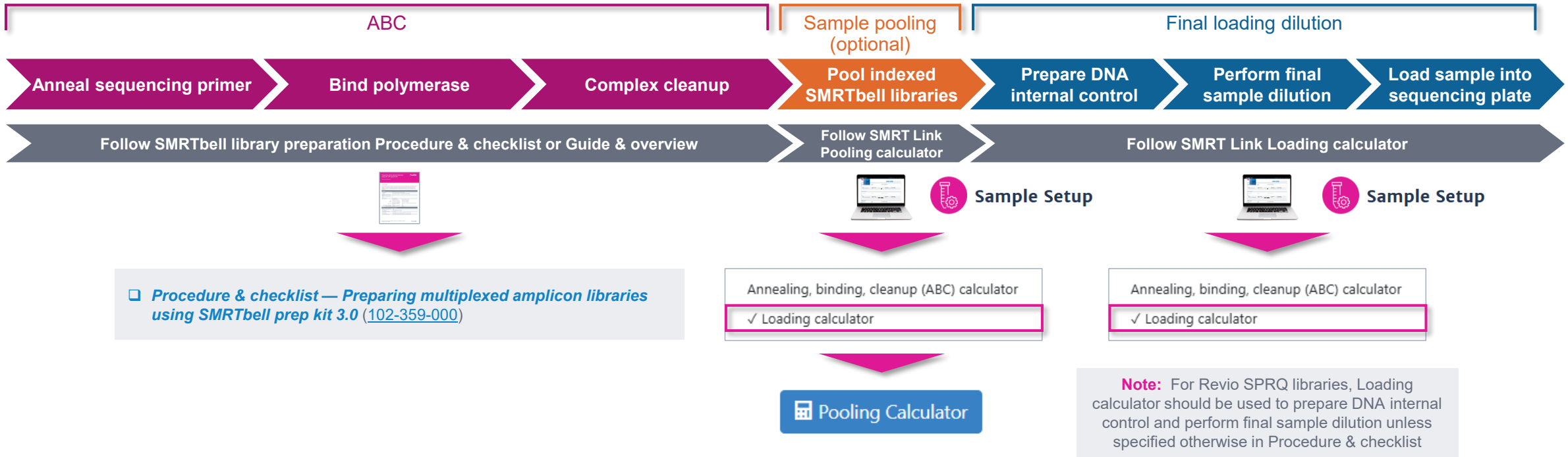
Note: If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator)

[1] If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator).

Sample setup workflow overview for Revio SPRQ polymerase libraries

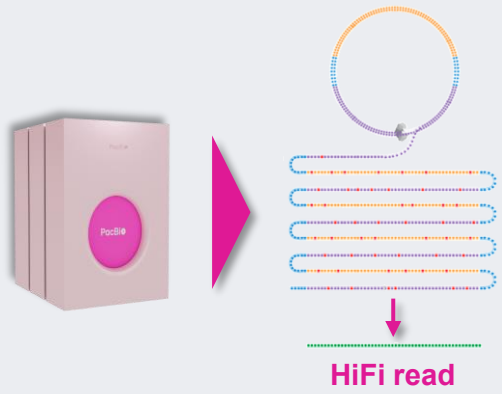
For binding libraries with Revio SPRQ polymerase kit, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1,2]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 amplicon library	**Revio SPRQ polymerase kit** (103-520-100)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator



Sample Setup & Run Design recommendations for SPK 3.0 multiplexed amplicon libraries – Vega system

Workflow	Key setup parameters	Vega system recommended settings
		Amplicon samples (0.5 kb to >10 kb)
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Vega polymerase kit
	Concentration on plate (OPLC)	300 – 400 pM
Run design	Application type	<3 kb amplicons / >=3 kb amplicons
	Library type	Standard
	Insert size	Specify an insert size (500 base pairs minimum)[1]
	Movie acquisition time	12 hrs (<5 kb) / 24 hrs (~5 – 20 kb)
	Use adaptive loading	N/A
	Data options[1]	Include base kinetics = NO Consensus Mode = MOLECULE[2]



Recommended target loading level range for Vega system is **~50 – 70%**

[1] The insert size is the length of the double-stranded nucleic acid fragment in a SMRTbell template, excluding the hairpin adapters. This matches the average insert size for the sample; the size range boundaries are described in the library preparation protocol. **Note:** If the library contains a mixture of multiple discrete-sized fragments, then enter the size of the shortest fragment to help produce more balanced loading of different fragment sizes in the sample.

[2] For PCR-indexed amplicon libraries ligated to standard (non-barcoded) SMRTbell adapters, specify **Sample is indexed = NO** in the SMRT Link run design and then manually perform sample demultiplexing in SMRT Link using Demultiplex Barcodes data utility.

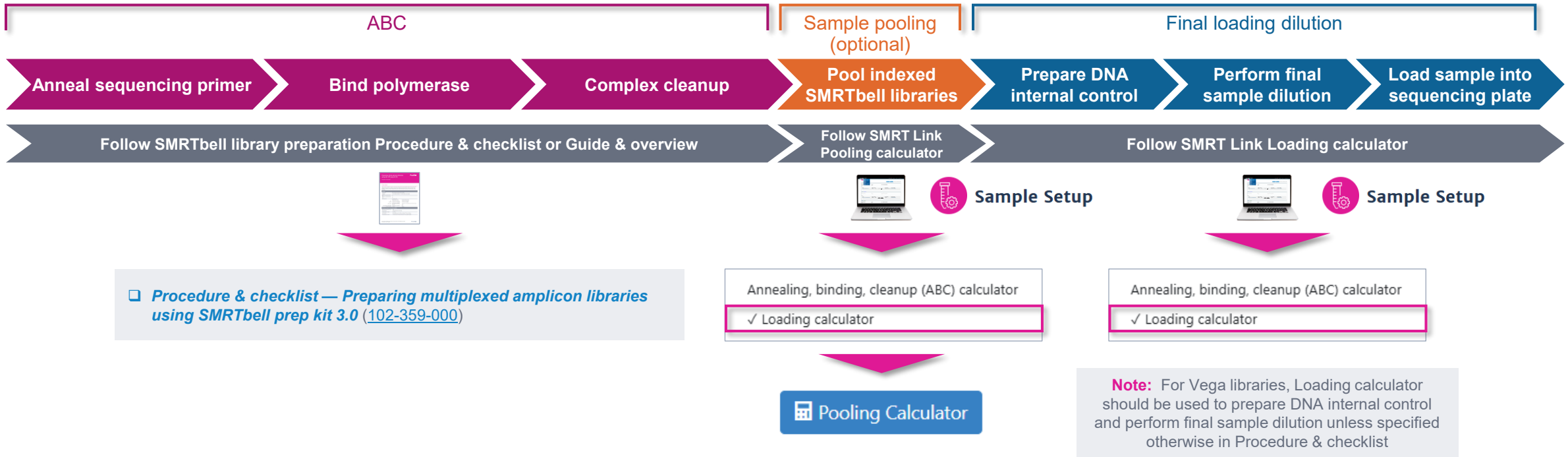
[3] **Note:** By default, Consensus mode is automatically set to **MOLECULE** for <3 kb amplicon samples and >=3 kb amplicon samples.

PacBio

Sample setup workflow overview for Vega polymerase libraries

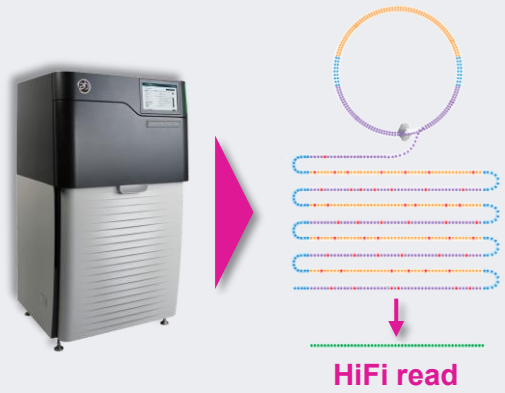
For binding libraries with Vega polymerase kit, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1,2]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 amplicon library	**Vega polymerase kit** (103-520-100)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator



Sample Setup & Run Design recommendations for SPK 3.0 multiplexed amplicon libraries – Sequel II/IIe system

Workflow	Key setup parameters	Vega system recommended settings
		Hybrid capture samples (~3 – 12 kb)
Sample setup	Library type	Standard
	Primer	Sequencing primer 3.2
	Polymerase kit	Sequel binding kit 3.2
	Concentration on plate (OPLC)	85 pM
Run design	Application type	<3 kb amplicons / >=3 kb amplicons
	SMRTbell Adapter Design	Overhang – SMRTbell prep kit 3.0
	Movie acquisition time	12 hrs (<5 kb) / 24 hrs (~5 – 20 kb)
	Use adaptive loading	YES
	Data options[1]	CCS Analysis Output - Include Low Quality Reads = NO CCS Analysis Output - Include Kinetics Information = NO Detect and Resolve Heteroduplex Reads = YES[2]



Recommended target *P1* loading range for Sequel IIe system is **~50 – 70%**

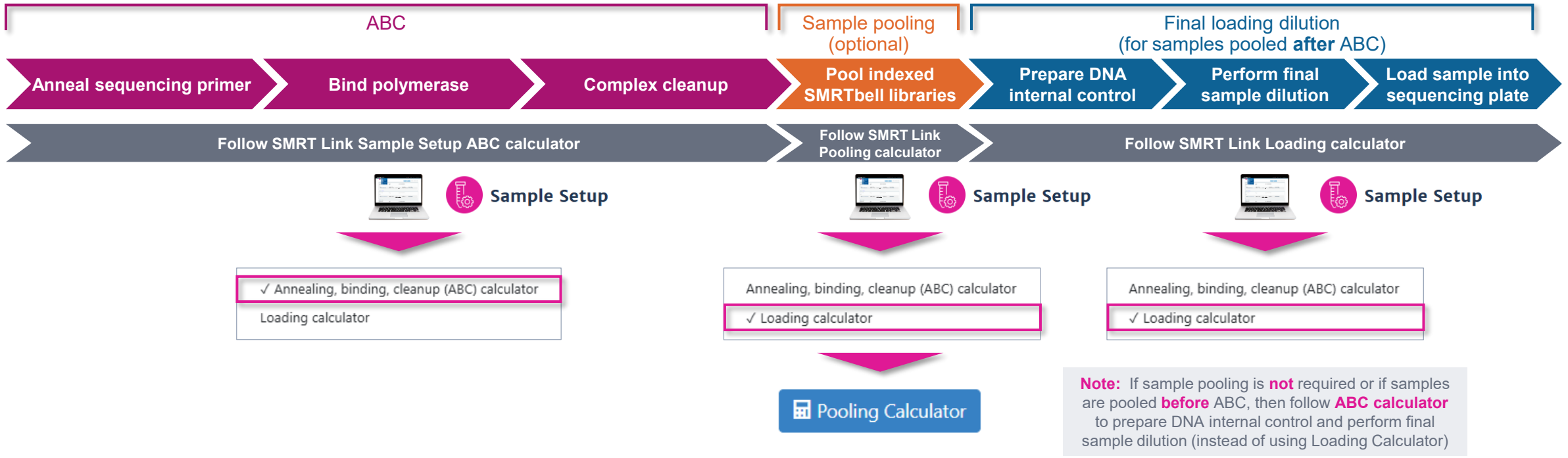
[1] For PCR-indexed amplicon libraries ligated to standard (non-barcoded) SMRTbell adapters, specify **Sample is Barcoded = NO** in the SMRT Link run design and then manually perform sample demultiplexing in SMRT Link using Demultiplex Barcodes data utility.

[2] **Note:** By default, heteroduplex detection and splitting feature is automatically set to **YES** for amplicon samples.

Sample setup workflow overview for Sequel II polymerase libraries

For binding libraries with Sequel II binding kit 3.1/3.2, follow SMRT Link Sample Setup ABC calculator instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 amplicon library	**Sequel II binding kit 3.1** (102-333-400); or **Sequel II binding kit 3.2** (102-333-300)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link Sample Setup ABC calculator
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator (if pooling samples **after** ABC)[1]



Note: If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator)

[1] If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator).

General best practices recommendations for preparing SPK 3.0 amplicon libraries for sequencing on Revio and Vega systems

Polymerase kit thawing procedure[1]

Revio polymerase kit / Revio SPRQ polymerase kit / Vega polymerase kit

Revio SPRQ polymerase kit
(103-520-100)

Vega polymerase kit
(103-517-600)

Thaw these reagents at room temperature		Keep these reagents on a cold block or on ice		Bring these reagents to room temperature 30 minutes prior to use	
	Annealing buffer		Sequencing polymerase		Loading buffer
	Standard sequencing primer		Sequencing control		SMRTbell cleanup beads
	Polymerase buffer				
	Loading buffer				
	Dilution buffer				

- Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Loading buffer should be left at room-temperature
- **Note:** Loading buffer is light sensitive and should be protected from light when not in use

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required sequencing preparation reagents.

PacBio

Multiplexed amplicon data analysis recommendations

Multiplexed amplicon data analysis recommendations for variant detection

Use SMRT Link and other analysis tools to fully characterize genetic complexity – structural variation, rare SNPs, indels, CNV, microsatellites, haplotypes, and phasing

General coverage recommendations for variant detection applications

- ≥50-fold HiFi read coverage per target locus is recommended for variant detection applications
- 6,000-fold HiFi read coverage per target locus is recommended for minor variant detection (1% sensitivity) applications

SMRT link Target Enrichment analysis application

- Use SMRT Link Target Enrichment analysis application to process and analyze multiplexed target enrichment samples including amplicons, hybrid capture, and PureTarget libraries
- The application includes the following main steps:
    1. (**Optional**) Mark PCR duplicate HiFi reads by sample using pbmarkdup.
    2. Align HiFi reads to the reference using pbmm2.
    3. (**Optional**) Call small variants using DeepVariant.
    4. (**Optional**) Phase small variants and haplotag BAMs using whatshap.
    5. (**Optional**) Call structural variants using pbsv.
    6. Produce target enrichment statistics.
- Files output for each sample include mapped BAM files, target enrichment statistics, and variant call sets
- **Note:** Sample demultiplexing must be performed **before** running the SMRT Link Target Enrichment workflow → To prepare input data for analysis, run the SMRT Link Demultiplex Barcodes data utility[1] by specifying an appropriate barcode set FASTA for demultiplexing
- Reference set (**required**): Specify a reference genome against which to align the reads and call variants (default set is Human Genome hg38, with Gencode v39 annotations)
- Target BED file (**required**): Specify a BED-format file[2] containing the target genes or regions of interest

PacBio

[1] See "Demultiplex Barcodes" in SMRT Link user guide for details on how to run the data utility.
[2] See "Appendix E – BED file format for PureTarget repeat expansion, Target Enrichment and HiFi Mapping applications" section in SMRT Link user guide for details about BED file format requirements.

SMRT link Target Enrichment analysis application is recommended for amplicon sequencing performance coverage QC evaluation

Recommended SMRT Link Target Enrichment analysis settings for different targeted sequencing applications

Targeted sequencing application	Target Enrichment analysis options			
	Include Fail Reads	Padding around regions (bp)	Mark PCR Duplicates	Variant Calling[1]
Amplicon sequencing	OFF (default)	0	OFF (default)	Optional
Hybrid capture sequencing[2]	OFF (default)	3000 (default)	ON	Optional
PureTarget sequencing (for PureTarget carrier screening & custom panels)	ON	0	OFF (default)	OFF (default)

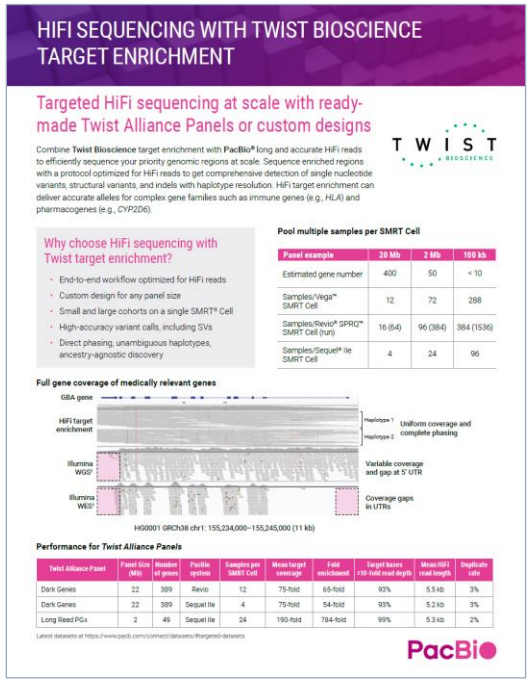
[1] To run this workflow with variant calling your SMRT Link server must be configured with Singularity. Please see the SMRT Link installation guide for additional details.

[2] **Note:** For QIAGEN hybrid capture long read panels, HiFi data analysis should be performed using QIAGEN software tools (e.g., QIAGEN GeneGlobe Data Analysis portal offers simplified downstream data analysis for HLA haplotyping and QIAGEN CLC Genomics Workbench is used to detect large structural variants in hereditary cancers).

Library preparation workflow overview for target enrichment sequencing using Twist Bioscience hybrid capture long read panels

Multiplexed HiFi target enrichment sequencing using Twist Bioscience hybrid capture long read panels: How to get started

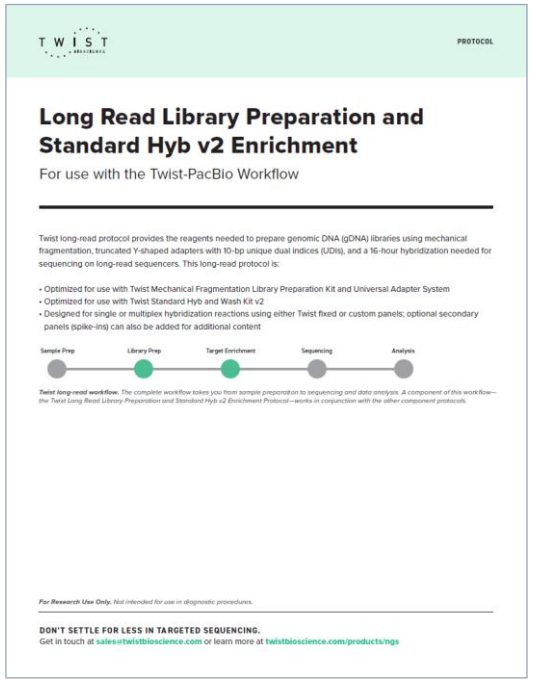
Application-specific educational literature



PacBio literature website (Link)

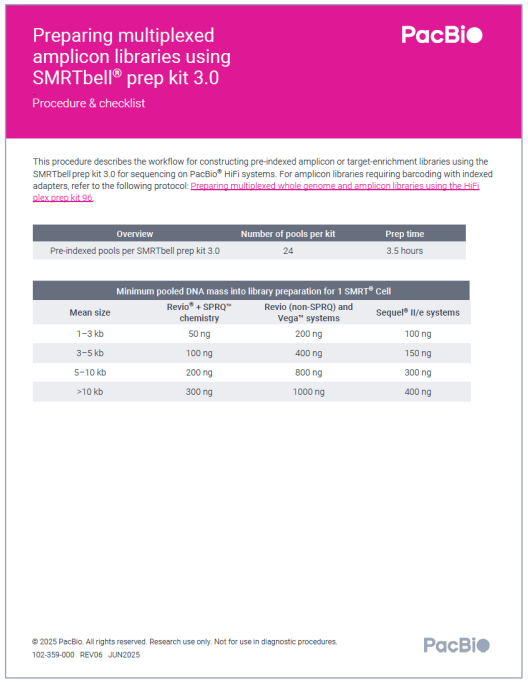
Application-specific brochures, informational guides and other product literature containing best practices recommendations for sample preparation and data analysis workflows.

Application-specific protocol documentation



Twist long read library preparation and standard hyb v2 enrichment (DOC-001320) [Twist Bioscience]

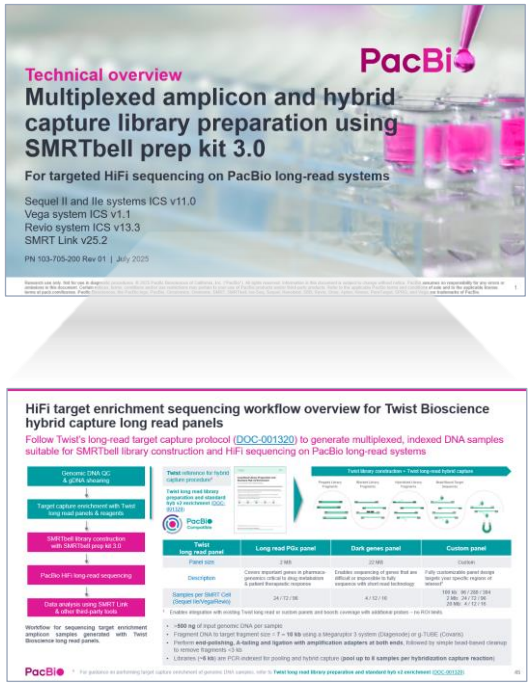
Technical documentation containing Twist target capture enrichment protocol details.



Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.

Application-specific technical overviews



Technical overview: Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0 (103-705-200)

Technical overview presentations describe the end-to-end library preparation, sequencing setup and data analysis workflow for specific applications. Example sequencing performance data are also summarized.

HiFi target enrichment sequencing workflow overview for Twist Bioscience hybrid capture long read panels

Follow Twist's long-read target capture protocol (DOC-001320) to generate multiplexed, indexed DNA samples suitable for SMRTbell library construction and HiFi sequencing on PacBio long-read systems

Genomic DNA QC & gDNA shearing

↓

Target capture enrichment with Twist long read panels & reagents

↓

SMRTbell library construction with SMRTbell prep kit 3.0

↓

PacBio HiFi long-read sequencing

↓

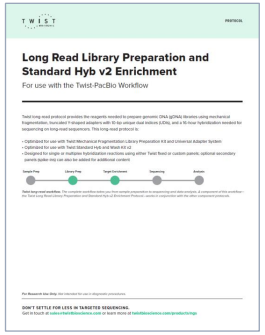
Data analysis using SMRT Link & other third-party tools

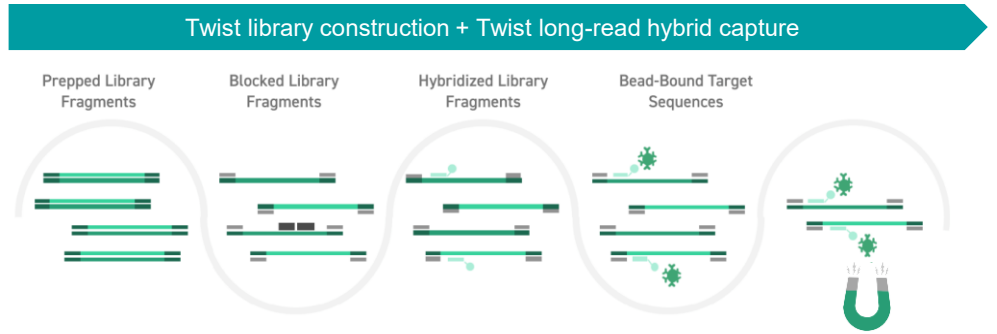
Workflow for sequencing target enrichment amplicon samples generated with Twist Bioscience long read panels.

Twist reference for hybrid capture procedure[1]

Twist long read library preparation and standard hyb v2 enrichment (DOC-001320)

PacBio Compatible





Twist long read panel	Long read PGx panel	Dark genes panel	Custom panel
Panel size	2 MB	22 MB	Custom
Description	Covers important genes in pharmaco-genomics critical to drug metabolism & patient therapeutic response	Enables sequencing of genes that are difficult or impossible to fully sequence with short read technology	Fully customizable panel design targets your specific regions of interest[1]
Samples per SMRT Cell (Sequel IIe/Vega/Revio)	24 / 72 / 96	4 / 12 / 16	100 kb: 96 / 288 / 384 2 Mb: 24 / 72 / 96 20 Mb: 4 / 12 / 16

[1] Enables integration with existing Twist long read or custom panels and boosts coverage with additional probes – no ROI limits.

- **>500 ng** of input genomic DNA per sample
- Fragment DNA to target fragment size = **7 – 10 kb** using a Megaruptor 3 system (Diagenode) or g-TUBE (Covaris)
- Perform **end-polishing, A-tailing and ligation with amplification adapters at both ends**, followed by simple bead-based cleanup to remove fragments <3 kb
- Libraries (**~5 kb**) are PCR-indexed for pooling and hybrid capture (**pool up to 8 samples per hybridization capture reaction**)

PacBio

[1] For guidance on performing target capture enrichment of genomic DNA samples, refer to **Twist long read library preparation and standard hyb v2 enrichment** (DOC-001320).

HiFi target enrichment sequencing workflow overview for Twist Bioscience hybrid capture long read panels (cont.)

Indexed hybrid capture DNA samples may be pooled for SMRTbell library construction by following PacBio *Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0* (102-359-000)

Genomic DNA QC
& gDNA shearing

↓

Target capture enrichment with Twist long read panels & reagents

↓

SMRTbell library construction with SMRTbell prep kit 3.0

↓

PacBio HiFi long-read sequencing

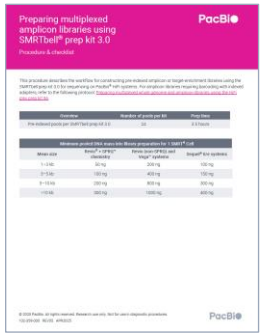
↓

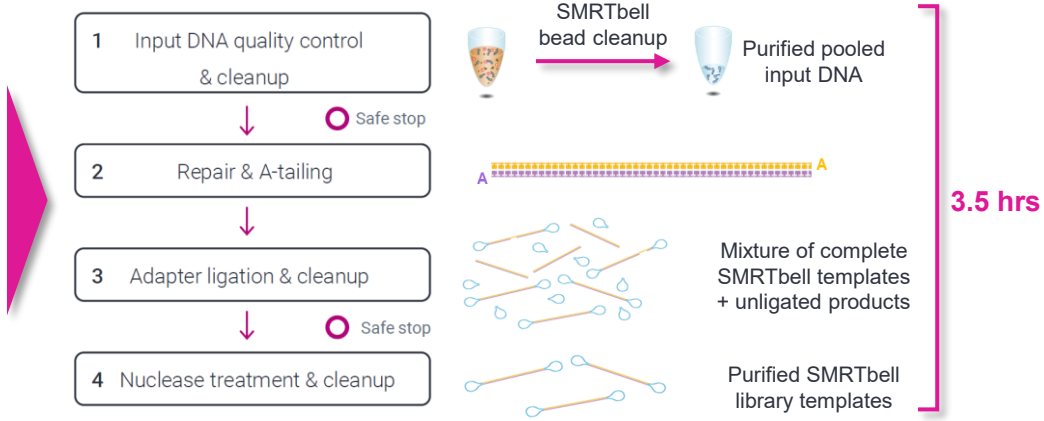
Data analysis using SMRT Link & other third-party tools

Workflow for sequencing target enrichment amplicon samples generated with Twist Bioscience long read panels.

PacBio reference for SMRTbell library construction procedure

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)





- Indexed hybrid capture DNA samples may be pooled for SMRTbell library construction by following PacBio Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0 (102-359-000)
- Multiplexed hybrid capture SMRTbell library may be sequenced on a single SMRT Cell using Sequel IIe, Vega or Revio system using 24-hour data acquisition (movie collection) time
- Sample demultiplexing can be performed manually in SMRT Link using the appropriate barcode set (e.g., Twist Universal Adapters with UDI barcode set)[1]
- HiFi data analysis can be performed using SMRT Link and other third-party tools:
  - SMRT Link Target enrichment analysis application workflow: Mark PCR duplicate HiFi reads; align HiFi reads to a reference; call small variants; phase small variants and haplotag BAMs; call structural variants; and produce coverage statistics

[1] Optionally configure SMRT Link run design to automatically perform sample demultiplexing in SMRT Link after the sequencing run is completed by setting **Sample is indexed = YES** and specifying the appropriate barcode set (e.g., Twist Universal Adapters with UDI barcode set).

Targeted sequencing performance for Twist Bioscience hybrid capture long read panels

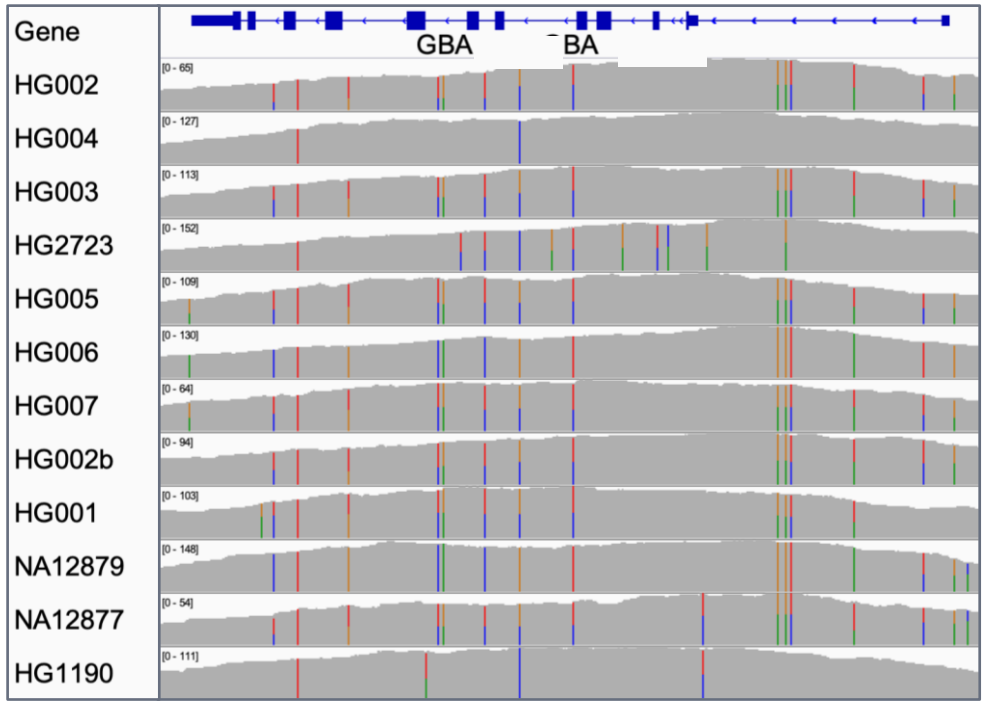
Example: Twist Alliance Dark Genes Panel

- Comprehensive 22 Mb panel: Full gene coverage for 389 **challenging medically-relevant genes**[1]
- Uncover genes in "**NGS dead zones**" that are difficult to sequence or map with short reads[2,3]

Dark Genes panel sequencing performance

	Sequel IIe system	Revio system[4]
Samples / SMRT Cell	4	12
HiFi yield / SMRT Cell	19.53 Gb	51.43 Gb
Mean read length	5.2 kb	5.5 kb
Median read quality	Q40	Q38
Mean reads / sample	893,459	724,795
Mean target coverage	75-fold	75-fold
Target bases ≥10-fold	93%	93%
Fold enrichment	54-fold	65-fold

Coverage at *GBA* for 12-plex on Revio system



[1] Ji *et al.* Characterizing the genetic polymorphisms in 370 challenging medically relevant genes using long-read sequencing data from 41 human individuals among 19 global populations. bioRXiv https://doi.org/10.1101/2022.08.03.502734.
[2] Mandelker *et al.* 2016. Navigating highly homologous genes in a molecular diagnostic setting: a resource for clinical next-generation sequencing. Genetics in Medicine.
[3] Wenger *et al.* 2019. Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. Nature Biotech.
[4] Example Revio data shown were generated using Revio non-SPRQ chemistry (see PacBio datasets website for list of genes included in the Twist Alliance Dark Genes panel). With Revio SPRQ chemistry, up to 16 hybrid capture samples for a 20 Mb panel size may be multiplexed on a single Revio SMRT Cell.

Targeted sequencing performance for Twist Bioscience hybrid capture long read panels (cont.)

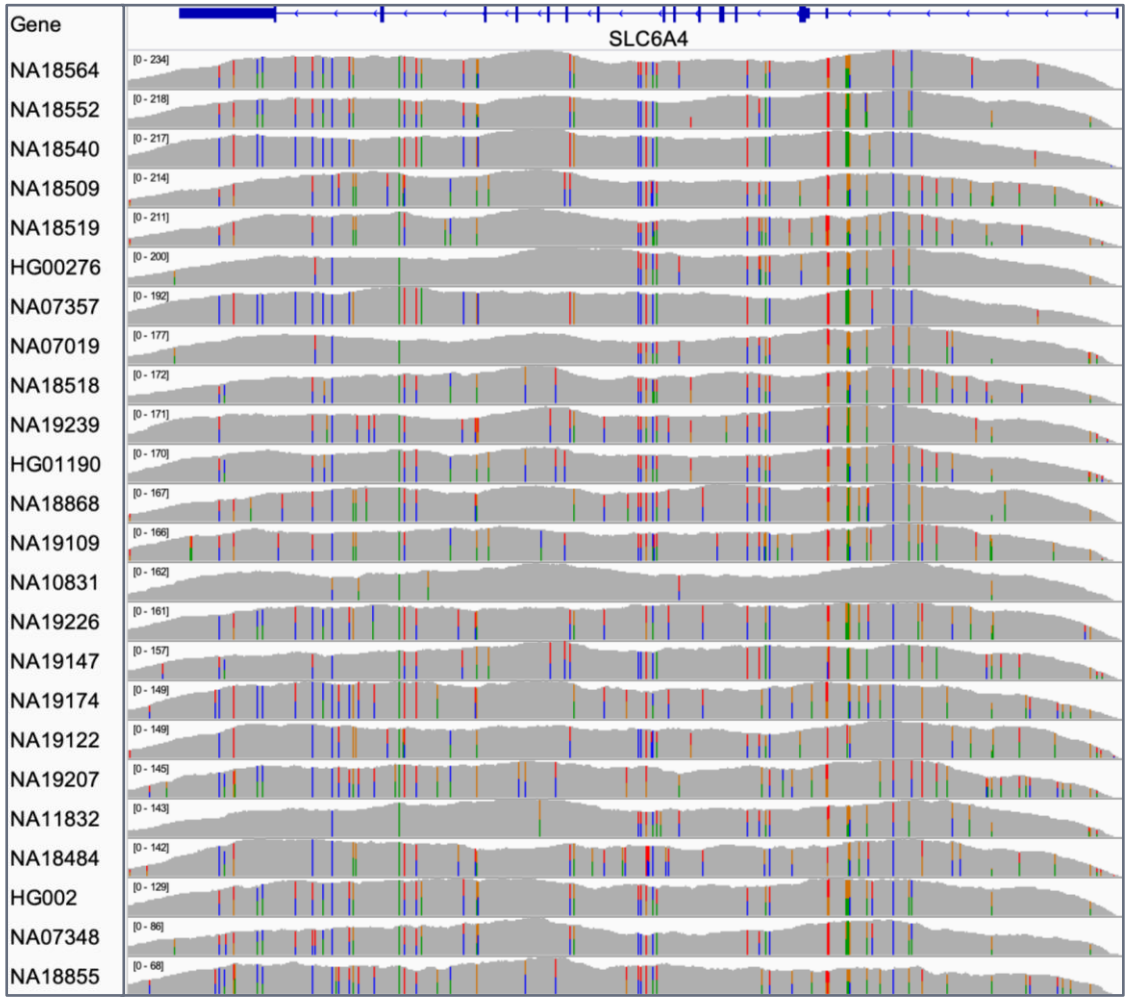
Example: Twist Alliance Long Read PGx Panel

- Comprehensive 50 gene 2 Mb panel
- 39 full-length genes enable phasing
- Includes all 20 genes with CPIC guidelines[1]

PGx panel sequencing performance for 24-plex of reference samples

	Sequel IIe system
Panel size	2 Mb
HiFi yield per SMRT Cell	20.11 Gb
Mean read length	5.3 kb
Mean reads per sample	149,749
Mean target coverage	190-fold
Target bases ≥20-fold	96%
Fold enrichment	784-fold
PCR duplicate rate	2%
Demultiplex yield	96%

Uniform coverage at *SLC6A4* across 24 samples



Full gene coverage + sample uniformity

[1] Twist Alliance Long Read PGx panel BED file is available for download from the Twist Bioscience website.

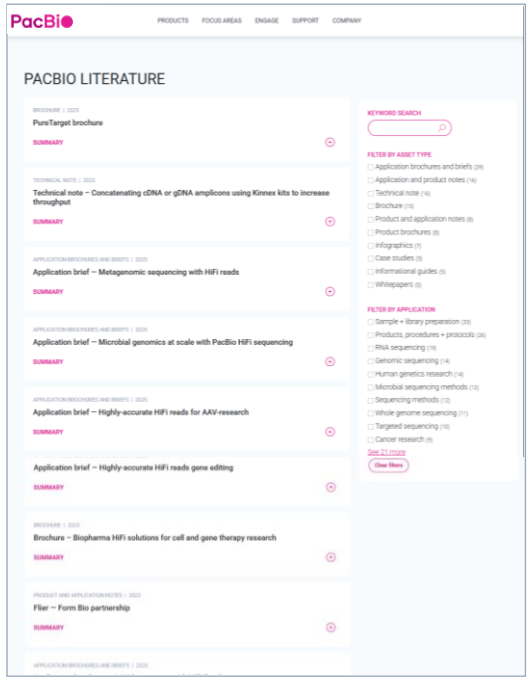
Library preparation workflow overview for target enrichment sequencing using QIAGEN hybrid capture long read panels

Multiplexed HiFi target enrichment sequencing using QIAGEN hybrid capture long read panels: How to get started

Application-specific educational literature

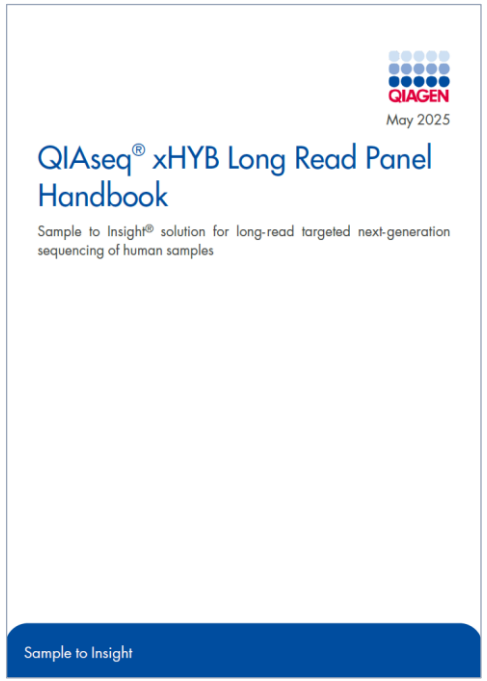
Application-specific protocol documentation

Application-specific technical overviews



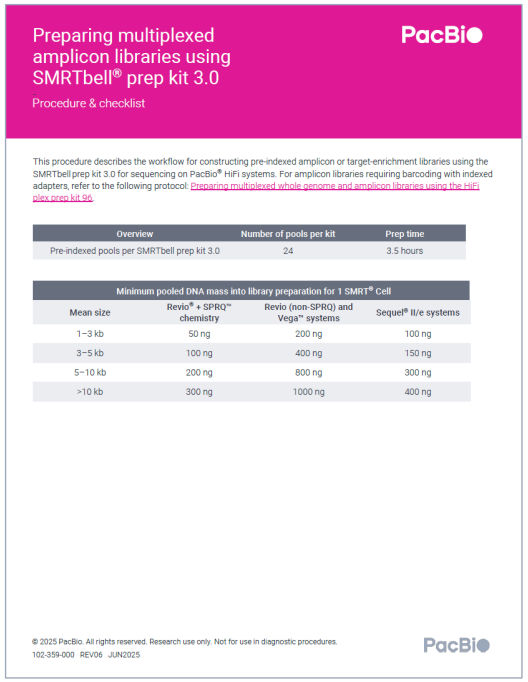
PacBio literature website (Link)

Application-specific brochures, informational guides and other product literature containing best practices recommendations for sample preparation and data analysis workflows.



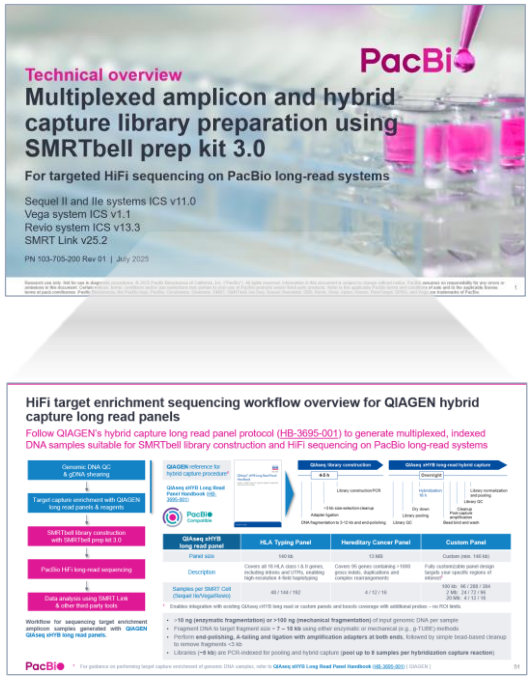
QIAseq xHYB Long Read Panel Handbook (HB-3695-001) [QIAGEN]

Technical documentation containing QIAGEN QIAseq xHYB long read panel hybrid capture protocol details.



Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000) [PacBio]

Technical documentation containing PacBio SMRTbell library construction details.



Technical overview: Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0 (103-705-200)

Technical overview presentations describe the end-to-end sample preparation, sequencing setup and data analysis workflow for specific applications. Example sequencing performance data are also summarized.

HiFi target enrichment sequencing workflow overview for QIAGEN hybrid capture long read panels

Follow QIAGEN's hybrid capture long read panel protocol (HB-3695-001) to generate multiplexed, indexed DNA samples suitable for SMRTbell library construction and HiFi sequencing on PacBio long-read systems

Genomic DNA QC & gDNA shearing

↓

Target capture enrichment with QIAGEN long read panels & reagents

↓

SMRTbell library construction with SMRTbell prep kit 3.0

↓

PacBio HiFi long-read sequencing

↓

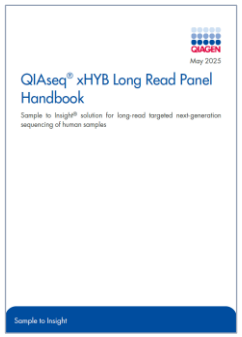
Data analysis using SMRT Link & other third-party tools

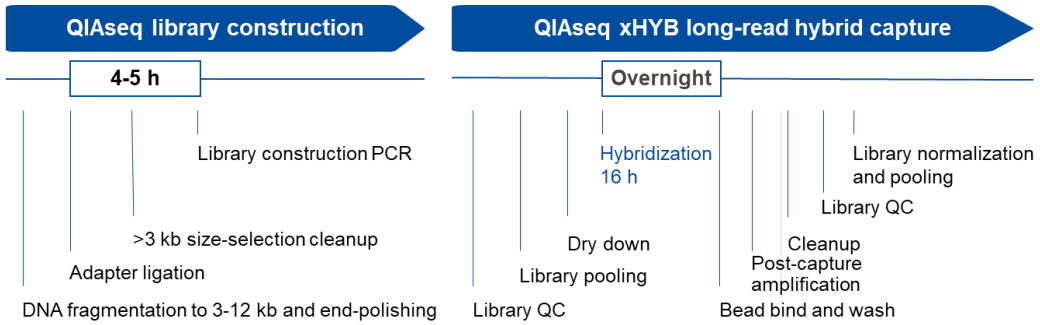
Workflow for sequencing target enrichment amplicon samples generated with QIAGEN QIAseq xHYB long read panels.

QIAGEN reference for hybrid capture procedure[1]

QIAseq xHYB Long Read Panel Handbook (HB-3695-001)

PacBio Compatible





QIAseq xHYB long read panel	HLA Typing Panel	Hereditary Cancer Panel	Custom Panel
Panel size	140 kb	13 MB	Custom (min. 140 kb)
Description	Covers all 18 HLA class I & II genes, including introns and UTRs, enabling high-resolution 4-field haplotyping	Covers 95 genes containing >1000 gross indels, duplications and complex rearrangements	Fully customizable panel design targets your specific regions of interest[1]
Samples per SMRT Cell (Sequel IIe/Vega/Revio)	48 / 144 / 192	4 / 12 / 16	100 kb: 96 / 288 / 384 2 Mb: 24 / 72 / 96 20 Mb: 4 / 12 / 16

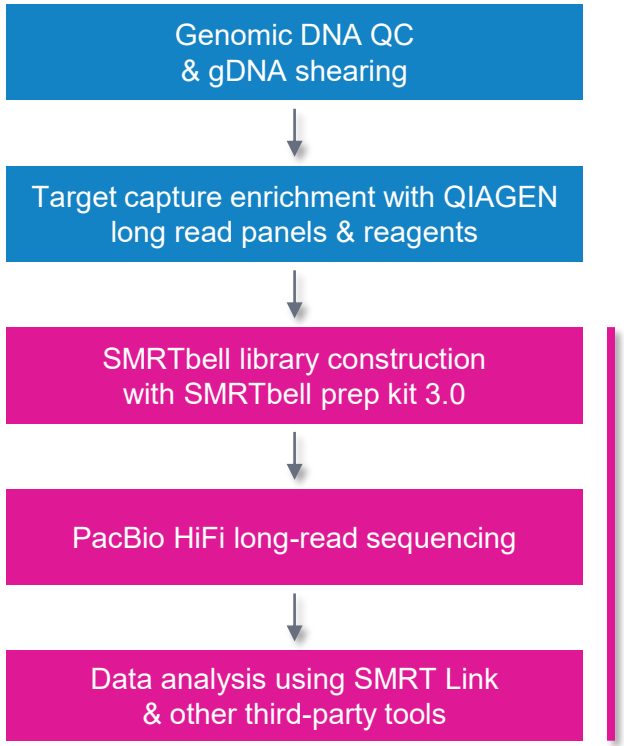
[1] Enables integration with existing QIAseq xHYB long read or custom panels and boosts coverage with additional probes – no ROI limits.

- **>10 ng (enzymatic fragmentation) or >100 ng (mechanical fragmentation)** of input genomic DNA per sample
- Fragment DNA to target fragment size = **7 – 10 kb** using either enzymatic or mechanical (e.g., g-TUBE) methods
- Perform **end-polishing, A-tailing and ligation with amplification adapters at both ends**, followed by simple bead-based cleanup to remove fragments <3 kb
- Libraries (**~5 kb**) are PCR-indexed for pooling and hybrid capture (**pool up to 8 samples per hybridization capture reaction**)

[1] For guidance on performing target capture enrichment of genomic DNA samples, refer to **QIAseq xHYB Long Read Panel Handbook** (HB-3695-001) [QIAGEN]

HiFi target enrichment sequencing workflow overview for QIAGEN hybrid capture long read panels (cont.)

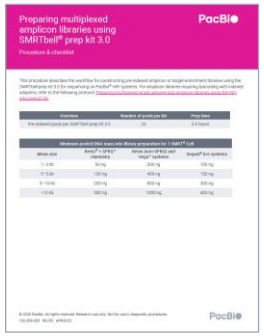
Indexed hybrid capture DNA samples may be pooled for SMRTbell library construction by following PacBio *Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0* (102-359-000)

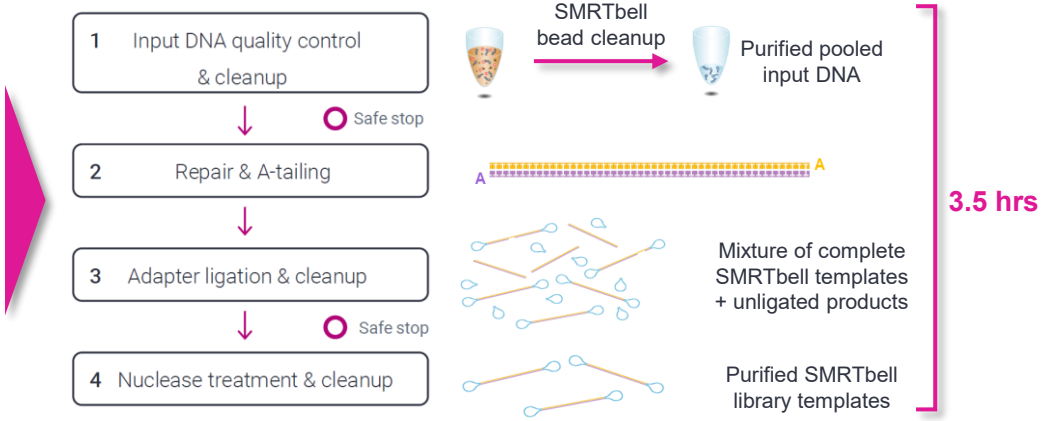


Workflow for sequencing target enrichment amplicon samples generated with QIAGEN QIAseq xHYB long read panels.

PacBio reference for SMRTbell library construction procedure

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)





- Indexed hybrid capture DNA samples may be pooled for SMRTbell library construction by following PacBio Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0 (102-359-000)
- Multiplexed hybrid capture SMRTbell library may be sequenced on a single SMRT Cell using Sequel IIe, Vega or Revio system using 24-hour data acquisition (movie collection) time
- Sample demultiplexing[1] and HiFi data analysis should be performed using QIAGEN software tools:
  - QIAGEN GeneGlobe Data Analysis portal offers simplified downstream data analysis for HLA haplotyping
  - QIAGEN CLC Genomics Workbench is used to detect large structural variants in hereditary cancers

[1] For QIAGEN hybrid capture samples, configure SMRT Link run design to specify **Sample is indexed = NO** and then manually perform sample demultiplexing using QIAGEN software tools.

Example targeted sequencing performance for QIAGEN hybrid capture long read panels

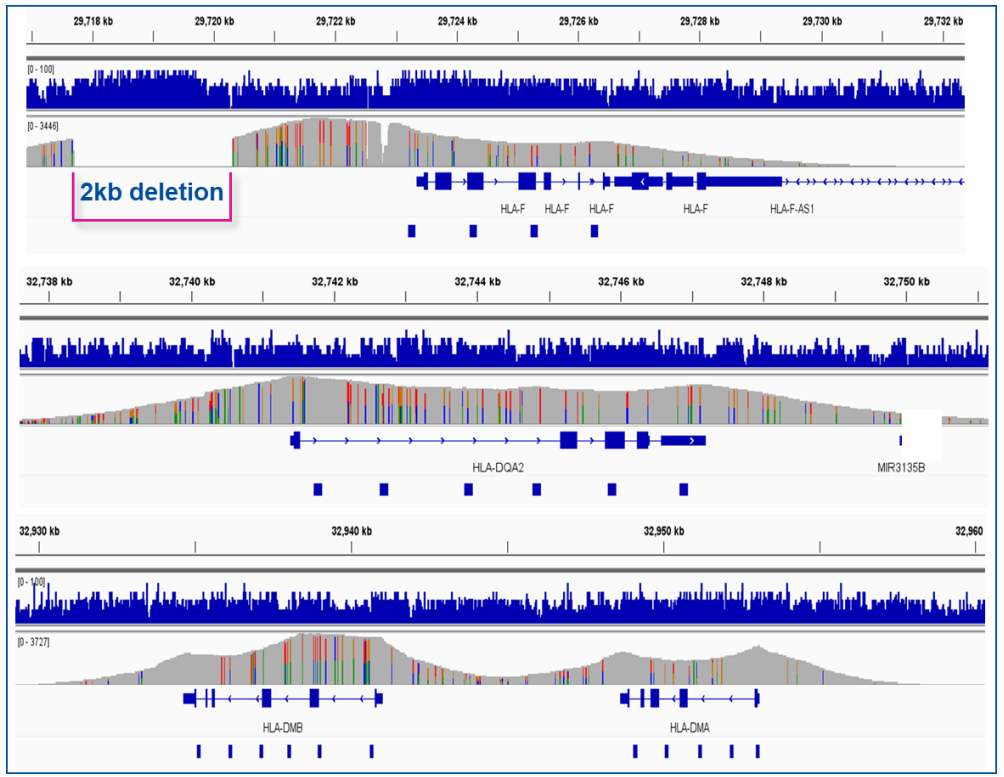
Example: QIAseq xHYB HLA Typing Panel

Long-read HLA typing panel covers all 18 HLA class I & II genes, including introns and UTRs

Long-read HLA panel targets 18 class I and class II HLA genes

Class I				Length
HLA-A	chr6	29941260	29945884	4624
HLA-B	chr6	31353875	31367067	13192
HLA-C	chr6	31268749	31272092	3343
HLA-E	chr6	30489509	30494194	4685
HLA-F	chr6	29722775	29726526	3751
HLA-G	chr6	29827825	29831021	3196
Class II				
HLA-DRA	chr6	32439887	32445046	5159
HLA-DRB1	chr6	32578775	32589848	11073
HLA-DRB5	chr6	32517353	32530287	12934
HLA-DQA1	chr6	32637406	32643684	6278
HLA-DQA2	chr6	32741391	32747198	5807
HLA-DQB1	chr6	32659467	32668383	8916
HLA-DQB2	chr6	32756098	32763532	7434
HLA-DOA	chr6	33004182	33009591	5409
HLA-DOB	chr6	32812767	32820466	7699
HLA-DMA	chr6	32948618	32953097	4479
HLA-DMB	chr6	32934636	32941028	6392
HLA-DPA1	chr6	33064569	33089696	25127

Haplotypes and structural variants are clearly detected in long-read HLA hybrid capture data[1]



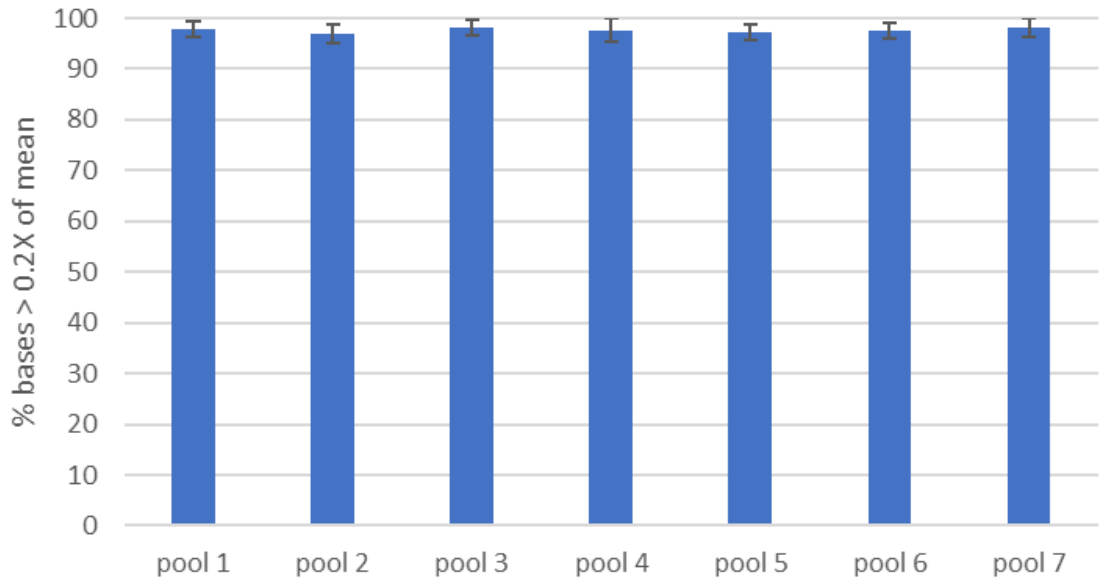
[1] Long-read HLA hybrid capture data shown were generated using a Revio system with non-SPRQ chemistry.

Example targeted sequencing performance for QIAGEN hybrid capture long read panels (cont.)

Example: QIAseq xHYB HLA Typing Panel

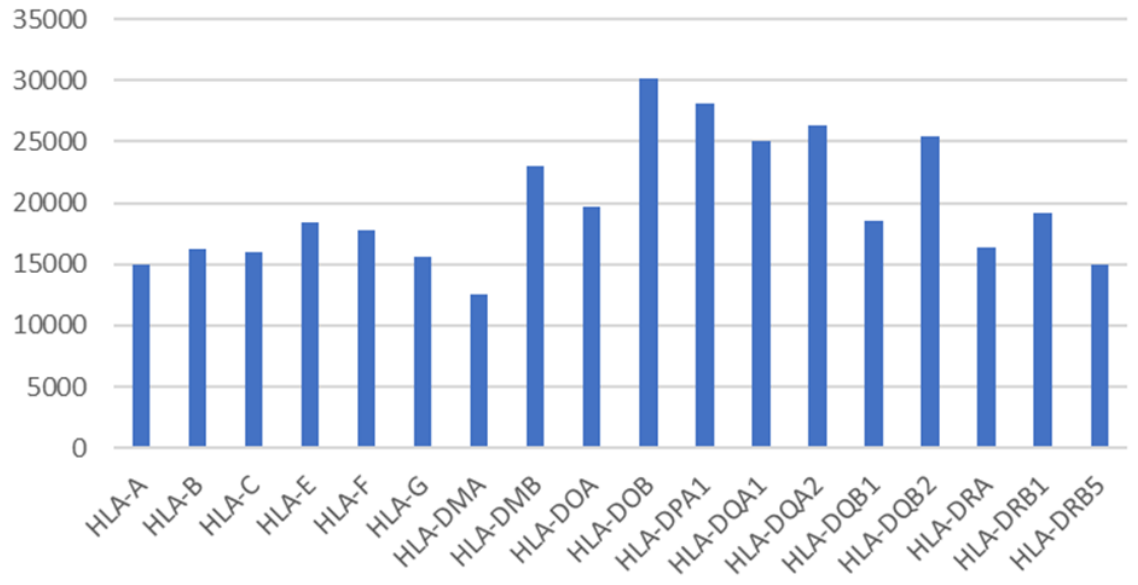
Long-read HLA typing panel produces uniform and deep coverage across 18 targeted class I & II HLA genes

HLA uniformity of coverage



99% of bases sequenced across all pooled samples[1] are covered at >0.2X of mean, indicating highly uniform coverage.

Coverage depth for 18 targeted HLA genes



Sequencing depth of coverage across all pooled samples[1] show good distribution of coverage depth over each HLA gene targeted.

[1] HLA typing libraries were constructed from different individuals with benchmarked haplotypes. Eight libraries were pooled together in one QIAseq xHYB hybrid capture reaction. Sequencing was performed on a Revio system with non-SPRQ chemistry.

Example targeted sequencing performance for QIAGEN hybrid capture long read panels (cont.)

Example: QIAseq xHYB HLA Typing Panel

Long-read HLA typing panel enables high-resolution 4-field haplotyping

Example: NA17129 HiFi HLA called haplotypes

HLA-A	*33:01:01:01/*74:01:01:01
HLA-B	*50:01:01/*53:01:01:01
HLA-C	*06:02:01/*07:18:01:01
HLA-DMA	*01:01:01/*01:01:01
HLA-DMB	*HLA-DMB/*01:03:01
HLA-DOA	*01:01:02:08/*01:01:02:45
HLA-DOB	*01:01:01:01/*01:01:01:23
HLA-DPB1	*01:01:01/*11:01:01
HLA-DPB2	*02:01/*02:01
HLA-DQA1	*01:03:01/*01:03:01:09
HLA-DQA2	*01:01:01/*01:01:01
HLA-DQB2	NoCall/*01:02:01:01
HLA-DRA	*01:01:01/*01:01:01:13
HLA-DRB1	*01:02:01:01/*HLA-DRB1
HLA-DRB3	*01:01:02/*01:01:02
HLA-E	*01:01:01/*01:01:01
HLA-F	*01:01:02/*01:03:01
HLA-G	*01:01:01:04/*01:03:01:02

Haplotyping with long reads provides clear resolution between individuals (only class I shown)

BioSample	HLA-A1	HLA-A1	HLA-B1	HLA-B1	HLA-C1	HLA-C1
NA09301	HLA-A*23:01:01:01	HLA-A*30:01:01:01	HLA-B*13:02:01:01	HLA-B*49:01:01:01	HLA-C*06:02:01:01	HLA-C*07:01:01:01
NA07439	HLA-A*29:02:01:01	HLA-A*68:02:01:01	HLA-B*42:02:01:02	HLA-B*53:01:01:01	HLA-C*04:01:01:01	HLA-C*17:01:01:02
NA12244	HLA-A*02:01:01:01	HLA-A*23:01:01:01	HLA-B*15:01:01:01	HLA-B*41:01:01:01	HLA-C*03:03:01:01	HLA-C*17:01:01:02
NA17057	HLA-A*24:20:01:01	HLA-A*33:03:01:01	HLA-B*15:11:01	HLA-B*44:03:01:01	HLA-C*03:04:01:01	HLA-C*14:03:01:01
NA17039	HLA-A*30:01:01:01	HLA-A*31:01:02:01	HLA-B*35:08:01:01	HLA-B*42:02:01:02	HLA-C*04:01:01:06	HLA-C*17:01:01:02
NA17119	HLA-A*03:01:01:01	HLA-A*74:01:01:08	HLA-B*35:01:01:01	HLA-B*42:02:01:02	HLA-C*07:18:08:01	HLA-C*17:01:01:10
NA17115	HLA-A*30:02:01:02	HLA-A*68:02:01:01	HLA-B*15:10:01:01	HLA-B*57:03:01:10	HLA-C*03:04:02:01	HLA-C*08:02:01:01
NA17130	HLA-A*01:01:01:01	HLA-A*02:01:01:01	HLA-B*08:01:01:01	HLA-B*14:02:01:01	HLA-C*07:01:01:01	HLA-C*08:02:01:01
NA17129	HLA-A*33:01:01:01	HLA-A*74:01:01:01	HLA-B*50:01:01:01	HLA-B*53:01:01:01	HLA-C*06:02:01:01	HLA-C*07:18:01:01
NA17210	HLA-A*26:01:01:01	HLA-A*66:01:01:01	HLA-B*38:01:01:01	HLA-B*38:01:01:01	HLA-C*12:03:01:01	HLA-C*12:03:01:33
NA17211	HLA-A*24:02:01:01	HLA-A*29:02:01:01	HLA-B*15:536	HLA-B*44:03:01:01	HLA-C*03:03:01:01	HLA-C*16:01:01:01
NA17212	HLA-A*02:05:01:01	HLA-A*11:01:01:01	HLA-B*27:02:01:01	HLA-B*52:01:01:01	HLA-C*02:02:02:03	HLA-C*12:02:02:01
NA17213	HLA-A*02:01:01:01	HLA-A*24:02:01:01	HLA-B*27:05:02:08	HLA-B*51:01:01:01	HLA-C*01:02:01:01	HLA-C*15:02:01:01
NA17229	HLA-A*24:02:01:01	HLA-A*31:01:02:01	HLA-B*35:01:01:01	HLA-B*35:20:01	HLA-C*03:04:01:01	HLA-C*04:01:01:01
NA17230	HLA-A*01:01:01:01	HLA-A*32:01:01:01	HLA-B*15:01:01:01	HLA-B*27:05:02:05	HLA-C*01:02:01:08	HLA-C*02:02:02:01
NA17269	HLA-A*02:01:01:01	HLA-A*26:01:01:01	HLA-B*40:01:02:01	HLA-B*40:02:01:01	HLA-C*02:02:02:01	HLA-C*03:04:01:01
NA17274	HLA-A*02:05:01:01	HLA-A*26:01:01:01	HLA-B*38:01:01:01	HLA-B*58:01:01:01	HLA-C*07:18:01:01	HLA-C*12:03:01:01
NA17282	HLA-A*03:01:01:01	HLA-A*11:01:01:01	HLA-B*35:03:01:01	HLA-B*56:01:01:02	HLA-C*01:02:01:01	HLA-C*04:01:01:01
NA17285	HLA-A*02:05:01:01	HLA-A*23:01:01:01	HLA-B*35:02:01:01	HLA-B*51:08:01:01	HLA-C*04:01:01:06	HLA-C*16:02:01:01
NA17084	HLA-A*11:01:01:01	HLA-A*80:01:01:02	HLA-B*38:02:02:01	HLA-B*40:02:01:01	HLA-C*04:01:01:01	HLA-C*07:02:01:01
NA17114	HLA-A*02:02:01:01	HLA-A*11:01:01:01	HLA-B*15:17:01:01	HLA-B*15:17:01:01	HLA-C*07:01:02:01	HLA-C*07:01:81
NA17115	HLA-A*30:02:01:02	HLA-A*68:02:01:01	HLA-B*15:10:01:01	HLA-B*57:03:01:10	HLA-C*03:04:02:01	HLA-C*08:02:01:01
NA17119	HLA-A*03:01:01:01	HLA-A*74:01:01:08	HLA-B*35:01:01:01	HLA-B*42:02:01:02	HLA-C*07:18:08:01	HLA-C*17:01:01:10
NA17286	HLA-A*02:01:01:01	HLA-A*03:01:01:01	HLA-B*14:02:01:01	HLA-B*45:01:01:01	HLA-C*06:02:01:03	HLA-C*08:02:01:01
NA17289	HLA-A*02:01:01:01	HLA-A*23:01:01:01	HLA-B*14:06:02	HLA-B*44:02:01:01	HLA-C*05:01:01:02	HLA-C*08:02:01:01
NA17290	HLA-A*11:01:01:01	HLA-A*26:01:01:01	HLA-B*07:02:01:01	HLA-B*57:01:01:01	HLA-C*06:02:01:01	HLA-C*12:03:01:01
NA17295	HLA-A*01:01:01:01	HLA-A*24:02:01:01	HLA-B*08:01:01:01	HLA-B*15:01:01:05	HLA-C*03:03:01:01	HLA-C*07:01:01:01

HiFiHLA[1] was used for calling haplotypes for all 18 gene targets, utilizing the IPD-IMGT/HLA database for allele reference

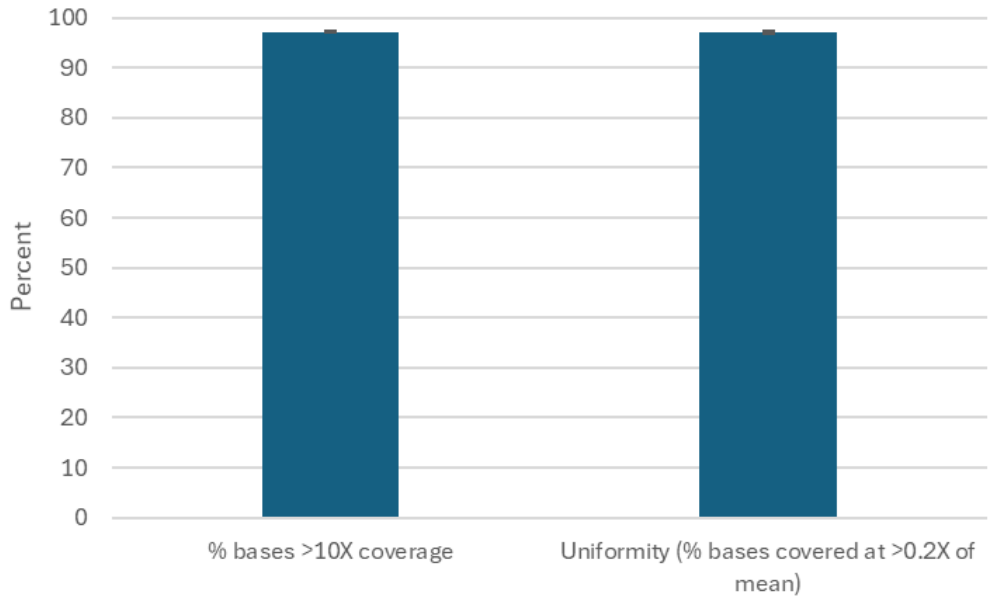
[1] Long-read HLA hybrid capture data shown were generated using a Revio system with non-SPRQ chemistry and analyzed with HiFiHLA. HiFi HLA is an HLA star-calling tool that generates high resolution (4-field) HLA allele calls from PacBio HiFi data. HiFiHLA identifies the closest matching allele(s) and any differences between a sample and the IPD-IMGT/HLA database. **Note:** HiFiHLA is deprecated and a newer phase-aware pharmacogenomic diplotyping tool for PacBio sequencing data (StarPhase) is available.

Example targeted sequencing performance for QIAGEN hybrid capture long read panels (cont.)

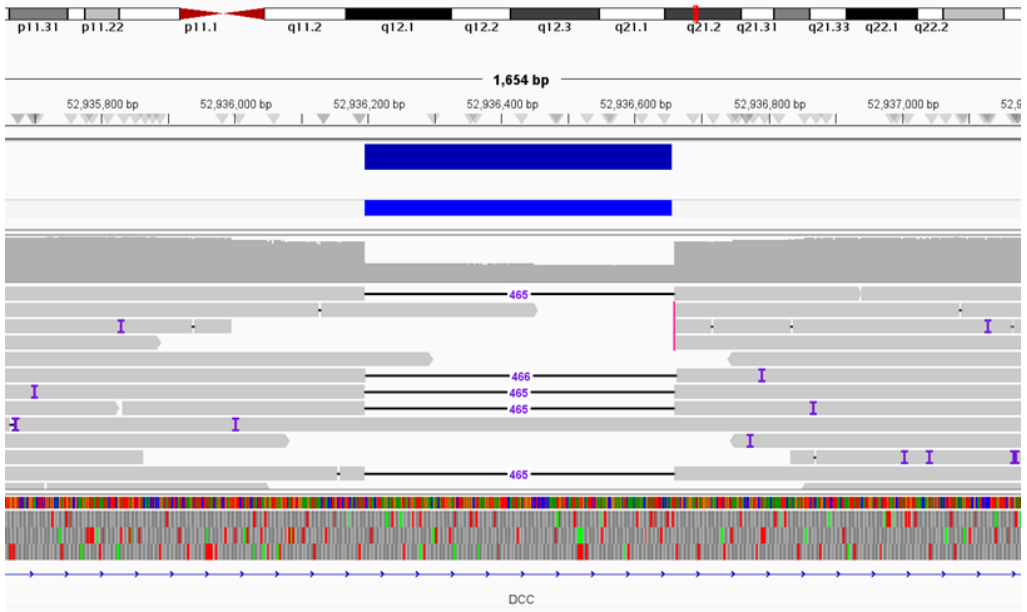
Example: QIAseq xHYB Long-Read Hereditary Cancer Panel

Hereditary cancer panel covers 95 genes containing >1000 gross indels, duplications and complex rearrangements

Hereditary cancer long-read capture panel produces highly uniform data



Hereditary cancer long-read capture panel detects a heterozygous 430 bp deletion in *DCC* gene implicated in colorectal cancer



Cancer panel probe design, amplification and capture chemistry ensure even, complete coverage over 95 targeted genes, including introns and upstream / downstream UTR sequences[1]

[1] Hereditary cancer long-read capture panel libraries were sequenced using a Revio system with non-SPRQ chemistry. Long-read mapping was performed with QIAGEN CLC Genomics Workbench. Structural variant detection was performed with Sniffles.

General recommendations for hybrid capture HiFi library preparation and sequencing

General SMRTbell library construction workflow for indexed target enrichment DNA samples generated with hybrid capture long read panels

Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0 (102-359-000)

Pool indexed hybrid capture DNA samples

↓

1.3X SMRTbell bead cleanup & DNA concentration QC

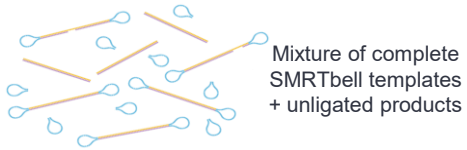
↓

2 hrs 15 min

DNA repair & A-tailing
40 min (Incl. 5 min hands on)

Standard (non-indexed) SMRTbell adapter

SMRTbell adapter ligation & 1.3X SMRTbell bead cleanup
55 min (Incl. 10 min hands on)

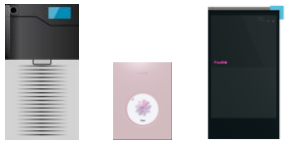


↓

Nuclease Treatment & 1.3X SMRTbell bead cleanup
40 min (Incl. 10 min hands on)

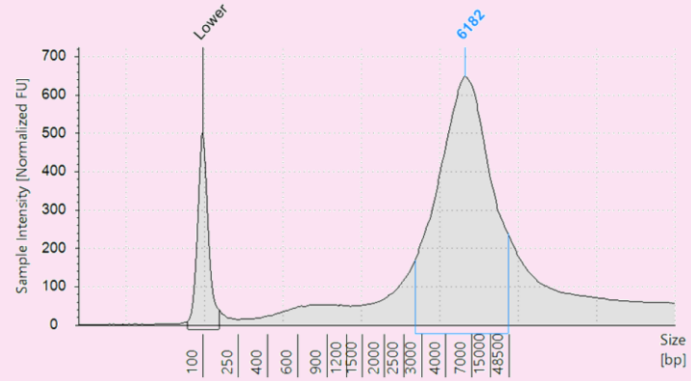
↓

Sequencing preparation & data collection



Mean size	Revio SPRQ system	Revio non-SPRQ / Vega system	Sequel IIe system
1 – 3 kb	50 ng	200 ng	100 ng
3 – 5 kb	100 ng	400 ng	150 ng
5 – 10 kb	200 ng	800 ng	300 ng
>10 kb	300 ng	1000 ng	400 ng

- Purify and concentrate pooled hybrid capture DNA using **1.3X SMRTbell cleanup beads**
- Perform multiplexed SMRTbell library construction with **SMRTbell prep kit 3.0** (102-182-700)
- Perform **DNA concentration QC and DNA sizing QC** analysis of final purified SMRTbell library



- Perform primer annealing, polymerase binding and complex cleanup (**ABC**) and final sample dilution
- **24-hour** movie collection time is recommended

[1] Lower total (pooled) input DNA amounts are possible but may result in lower sequencing yield if there is not enough SMRTbell library available for optimal SMRT Cell loading. Refer to ***Procedure & checklist – Multiplexed amplicon library preparation using SMRTbell Prep Kit 3.0*** (102-359-000) for up-to-date guidance on recommended DNA input amounts for SMRTbell library construction.

Genomic DNA quality requirements and DNA shearing recommendations for hybrid capture libraries[1]

Genomic DNA quality requirements

- We recommend DNA extraction methods that result in high molecular weight DNA with 70% of molecules larger than 10 kb and free of RNA
- PacBio Nanobind DNA extractions kits are a good option to ensure there is sufficient mass and quality of high molecular weight DNA for hybrid capture sequencing applications

DNA shearing recommendations

- Target shear lengths refer to the mean or modal size of the distribution
- The recommended size for shearing samples prior to target enrichment is 7–10 kb in order to maximize capture efficiency and HiFi read lengths
- Use any supported DNA shearing method (e.g., Megaruptor, Covaris g-TUBE, MiniG or enzymatic fragmentation)
  - Refer to manufacturer's instructions for the Diagenode Megaruptor and Covaris g-TUBE for recommendations on fragmentation settings for preferred DNA fragment sizes
  - Refer to PacBio documentation for recommended DNA shearing parameters for 1600 MiniG (SPEX Sample Prep)
  - Refer to appropriate PacBio Compatible partner or other third-party documentation if performing DNA shearing using other methods (e.g., seqWell LongPlex Long Fragment Multiplex Kit)

PacBio

[1] Refer to ***Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0*** (102-359-000) for the most up-to-date guidance on genomic DNA quality requirements and DNA shearing recommendations for hybrid capture libraries.

Sample multiplexing guidance for hybrid capture libraries

Capture pools consisting of 4–8 indexed libraries should be QC'ed and combined in equal mass prior to beginning SMRTbell library prep using the SMRTbell prep kit 3.0 (SPK 3.0)

- It is important to only combine samples from the same targeted panel into a SMRTbell library to ensure uniform coverage across samples
- The table below lists recommendations for the number of samples and capture pools per SMRT Cell on Vega, Revio, and Sequel II systems[1] and also shows the recommended DNA quantities per post-capture pool
- In general, we recommend aiming for 50-fold or greater HiFi coverage read depth for target regions

Recommended number of samples and capture pools to load per SMRT Cell on Vega, Revio, and Sequel II systems.

Revio system with SPRQ chemistry				
Panel target size	**Samples per SMRT Cell**	**Capture pools per SPK 3.0 library[2]**	**Samples per capture pool**	**DNA quantity per capture pool**
20 Mb	16	2	8	150 ng
2 Mb	96	12	8	33 ng
100 kb	384	48	8	8 ng

Revio system with non-SPRQ chemistry and Vega system chemistry				
Panel target size	**Samples per SMRT Cell**	**Capture pools per SPK 3.0 library[2]**	**Samples per capture pool**	**DNA quantity per capture pool**
20 Mb	12	2	6	200 ng
2 Mb	72	9	8	45 ng
100 kb	288	36	8	12 ng

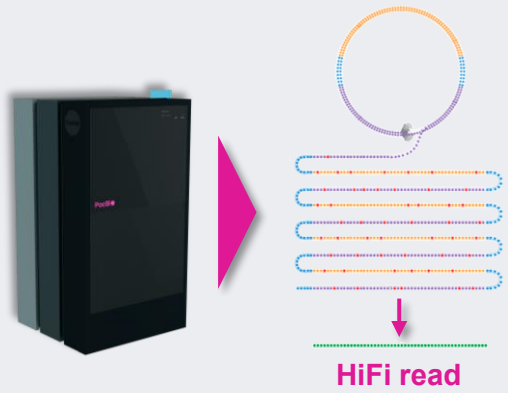
Sequel II/IIe system chemistry				
Panel target size	**Samples per SMRT Cell**	**Capture pools per SPK 3.0 library[2]**	**Samples per capture pool**	**DNA quantity per capture pool**
20 Mb	4	1	4	200 ng
2 Mb	24	3	8	67 ng
100 kb	96	12	8	17 ng

[1] The recommended sample multiplexing levels are intended to maximize the number of samples that can be run for each panel on a SMRT Cell. When pooling fewer samples than recommended, HiFi yield and sample loading level (*P1*) may be suboptimal even though the recommended per-sample HiFi read depth may be achieved.

[2] This guidance assumes capture pools consist of 8 samples per pool except for the 20 Mb panel on Vega/Revio non-SPRQ with 6 samples per pool and on the Sequel II system where 4 samples are in the pool. Refer to ***Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell Prep Kit 3.0*** (102-359-000) for the most up-to-date guidance on sample multiplexing.

Sample Setup & Run Design recommendations for SPK 3.0 hybrid capture libraries – Revio system

Workflow	Key setup parameters	Revio system recommended settings
		Hybrid capture samples (~3 – 12 kb)
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Revio (non-SPRQ) polymerase kit / Revio SPRQ polymerase kit
	Concentration on plate (OPLC)	300 – 400 pM
Run design	Application type	Hybrid capture
	Library type	Standard
	Movie acquisition time	24 hrs
	Use adaptive loading	YES
	Data options[1]	Include base kinetics = NO Consensus Mode = MOLECULE[2]



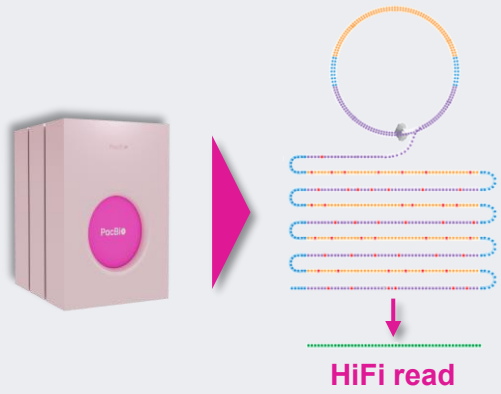
Recommended target *P1* loading range for Revio system is **~50 – 70%**

[1] For Twist Bioscience hybrid capture samples, optionally configure SMRT Link run design to automatically perform sample demultiplexing in SMRT Link after the sequencing run is completed by setting **Sample is indexed = YES** and specifying the appropriate barcode set (e.g., Twist Universal Adapters with UDI barcode set). For QIAGEN hybrid capture samples, configure SMRT Link run design to specify **Sample is indexed = NO** and then manually perform sample demultiplexing using QIAGEN software tools.

[2] **Note:** By default, Consensus mode is automatically set to **MOLECULE** for hybrid capture samples.

Sample Setup & Run Design recommendations for SPK 3.0 hybrid capture libraries – Vega system

Workflow	Key setup parameters	Vega system recommended settings
		Hybrid capture samples (~3 – 12 kb)
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Vega polymerase kit
	Concentration on plate (OPLC)	300 – 400 pM
Run design	Application type	Hybrid capture
	Library type	Standard
	Movie acquisition time	24 hrs
	Use adaptive loading	N/A
	Data options[1]	Include base kinetics = NO Consensus Mode = MOLECULE[2]



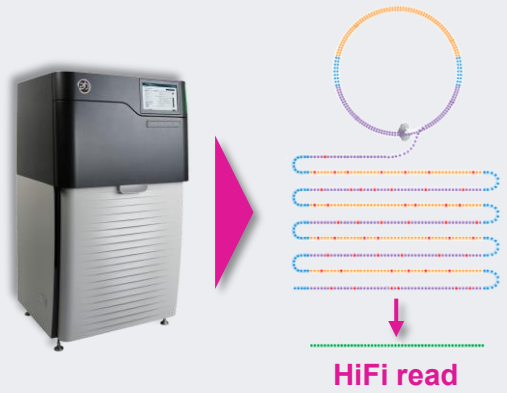
Recommended target loading level range for Vega system is **~50 – 70%**

[1] For Twist Bioscience hybrid capture samples, optionally configure SMRT Link run design to automatically perform sample demultiplexing in SMRT Link after the sequencing run is completed by setting **Sample is indexed = YES** and specifying the appropriate barcode set (e.g., Twist Universal Adapters with UDI barcode set). For QIAGEN hybrid capture samples, configure SMRT Link run design to specify **Sample is indexed = NO** and then manually perform sample demultiplexing using QIAGEN software tools.

[2] **Note:** By default, Consensus mode is automatically set to **MOLECULE** for hybrid capture samples.

Sample Setup & Run Design recommendations for SPK 3.0 hybrid capture libraries – Sequel II/IIe system

Workflow	Key setup parameters	Vega system recommended settings
		Hybrid capture samples (~3 – 12 kb)
Sample setup	Library type	Standard
	Primer	Sequencing primer 3.2
	Polymerase kit	Sequel binding kit 3.2
	Concentration on plate (OPLC)	85 pM
Run design	Application type	HiFi target enrichment
	SMRTbell Adapter Design	Overhang – SMRTbell prep kit 3.0
	Movie acquisition time	24 hrs
	Use adaptive loading	YES
	Data options[1]	CCS Analysis Output - Include Low Quality Reads = NO CCS Analysis Output - Include Kinetics Information = NO Detect and Resolve Heteroduplex Reads = YES[2]



Recommended target *P1* loading range for Sequel IIe system is **~50 – 70%**

[1] For Twist Bioscience hybrid capture samples, optionally configure SMRT Link run design to automatically perform sample demultiplexing in SMRT Link after the sequencing run is completed by setting **Sample is indexed = YES** and specifying the appropriate barcode set (e.g., Twist Universal Adapters with UDI barcode set). For QIAGEN hybrid capture samples, configure SMRT Link run design to specify **Sample is indexed = NO** and then manually perform sample demultiplexing using QIAGEN software tools.

[2] **Note:** By default, heteroduplex detection and splitting feature is automatically set to **YES** for HiFi target enrichment samples.

SMRT link Target Enrichment analysis application is recommended for amplicon sequencing performance coverage QC evaluation

Recommended SMRT Link Target Enrichment analysis settings for different targeted sequencing applications

Targeted sequencing application	Target Enrichment analysis options			
	Include Fail Reads	Padding around regions (bp)	Mark PCR Duplicates	Variant Calling[1]
Amplicon sequencing	OFF (default)	0	OFF (default)	Optional
Hybrid capture sequencing[2]	OFF (default)	3000 (default)	ON	Optional
PureTarget sequencing (for PureTarget carrier screening & custom panels)	ON	0	OFF (default)	OFF (default)

[1] To run this workflow with variant calling your SMRT Link server must be configured with Singularity. Please see the SMRT Link installation guide for additional details.

[2] **Note:** For QIAGEN hybrid capture long read panels, HiFi data analysis should be performed using QIAGEN software tools (e.g., QIAGEN GeneGlobe Data Analysis portal offers simplified downstream data analysis for HLA haplotyping and QIAGEN CLC Genomics Workbench is used to detect large structural variants in hereditary cancers).

PacBio

Technical documentation & applications support resources

Technical resources for multiplexed amplicon and hybrid capture library preparation, sequencing & data analysis

Sample & library preparation literature

- Application brief – HiFi target enrichment – Best practices (102-326-515)
- Application brief – Targeted sequencing for amplicons – Best practices (102-193-603)
- Application note – HiFi amplicon sequencing for pharmacogenetics: CYP2D6 (102-326-548)
- Application note – Targeted HiFi sequencing for congenital adrenal hyperplasia (102-326-547)
- Application note – Targeted HiFi sequencing for thalassemia (102-326-551)
- Hybrid capture protocol – QIAGEN – QIAseq xHYB Long Read Panel Handbook (HB-3695-001)
- Hybrid capture protocol – Twist Bioscience – Long Read Library Preparation and Standard Hyb v2 Enrichment (DOC-001320)
- Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000)
- Technical note – Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)
- Technical note – Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)
- Technical overview – Multiplexed amplicon and hybrid capture library preparation using SMRTbell prep kit 3.0 (103-705-200)

Data analysis resources

- SMRT Link Cloud v25.3 user guide (103-720-300)
- SMRT Link v25.3 user guide (103-720-100)
- SMRT Link web services API use cases (103-653-100)
- SMRT Tools reference guide (103-653-200)

Technical resources for multiplexed amplicon and hybrid capture library preparation, sequencing & data analysis (cont.)

Posters

- ESHG poster (2023): From sample to star alleles: a long-read pharmacogenomics pipeline powered by Twist target enrichment and PacBio HiFi sequencing [Link]
- ACMG poster (2023): Highly scalable pharmacogenomic panel testing with hybrid capture and long-read sequencing [Link]
- ESHG poster (2022): Enablement of long-read targeted sequencing panels using Twist hybrid capture and PacBio HiFi sequencing [Link]

Publications

- Charnaud, S. et al. (2022) PacBio long-read amplicon sequencing enables scalable high-resolution population allele typing of the complex CYP2D6 locus. Commun. Biol. 25;5(1):168. [Link]
- Van der Lee, Maaike et al. (2022) Design and performance of a long-read sequencing panel for pharmacogenomics. BioRxiv preprint. [Link]
- Luo, Shiqiang et al. (2022) Detection of four rare thalassemia variants using single-molecule real time sequencing. Front. Genet. [Link]
- Twesigomwe, David et al. (2022) Characterization of CYP2D6 Pharmacogenetic variation in sub-Saharan African populations. Clin Pharmacol Ther. [Link]
- Scott, Erick R et al. (2022) Long-read HiFi sequencing of*NUDT15*: Phased full-gene haplotyping and pharmacogenomic allele discovery. Human Mutation. 43: 1557-1566 [Link]
- Te Paske, Iris B.A.W. et al. (2022) Non-coding aberrations in mismatch repair genes underlie a substantial part of the missing heritability in Lynch syndrome. Gastroenterology. [Link]
- Liu, Y et al. (2022) Comprehensive analysis of congenital adrenal hyperplasia using long-read sequencing. Clinical Chemistry. 68(7):927–939. [Link]

Technical resources for multiplexed amplicon and hybrid capture library preparation, sequencing & data analysis (cont.)

Webinars

- QIAGEN webinar (2025): Navigating genomic complexities with long-read sequencing in cancer research [Link]
- PacBio PRISM webinar (2025): Closing the gap: Solving complex medically relevant genes at scale [Link]
- PacBio PRISM webinar (2025): Implementing long-read HiFi sequencing: Amplicon, panel, and genome sequencing [Link]
- PacBio ASHG webinar (2021): Using HiFi reads for improved and accurate haplotyping and phasing of pharmacogenomic alleles [Link]

Example PacBio data sets

Targeted sequencing application	Dataset	Data type	PacBio system
PCR amplicon sequencing	CYP2D6 amplicon for PGx reference samples	HiFi long read	Sequel IIe System
HiFi hybrid capture target enrichment	Twist Alliance Dark Genes Panel	HiFi long read	Revio system
HiFi hybrid capture target enrichment	Twist Alliance Long Read PGx Panel for 10 reference samples	HiFi long read	Sequel IIe System
HiFi hybrid capture target enrichment	Twist Alliance Dark Gene Panel for NA12878	HiFi long read	Sequel IIe System
HiFi hybrid capture target enrichment	Twist Custom BRCA1/2 Panel	HiFi long read	Sequel IIe System

Visit our Datasets website for a complete list of example SMRT sequencing datasets for specific applications.

www.pacb.com

Revision history (description)	Version	Date
Initial release	01	November 2025