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Technical Overview: Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0

Sequel System ICS v8.0 / Sequel Chemistry 3.0 / SMRT Link v9.0
Sequel II System ICS v9.0 / Sequel II Chemistry 2.0 / SMRT Link v9.0
Sequel IIe System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0

PN 101-814-300 Ver 2021-02-01-B (March 2021)

Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0

1. Multiplexed Amplicon Library Sample Preparation Workflow Overview
2. Amplicon Sample QC Requirements and SMRTbell Express TPK 2.0 Handling Recommendations
3. Multiplexed Amplicon Library Preparation Using PacBio Barcoded Overhang Adapters (BOA)
4. Multiplexed Amplicon Library Preparation Using PacBio Barcoded Universal Primers (BUP)
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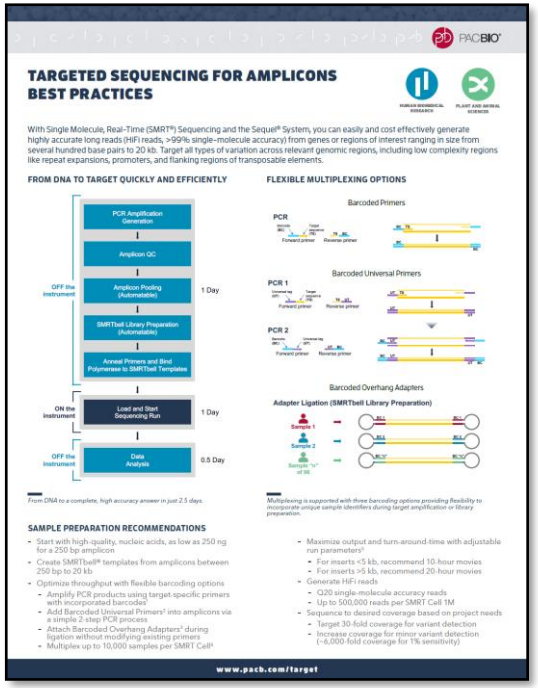
Appendix 1: Multiplexed Amplicon Library Preparation Using PacBio Barcoded M13 Primers

Appendix 2: Sample Preparation Recommendations for HLA Amplicon SMRT Sequencing

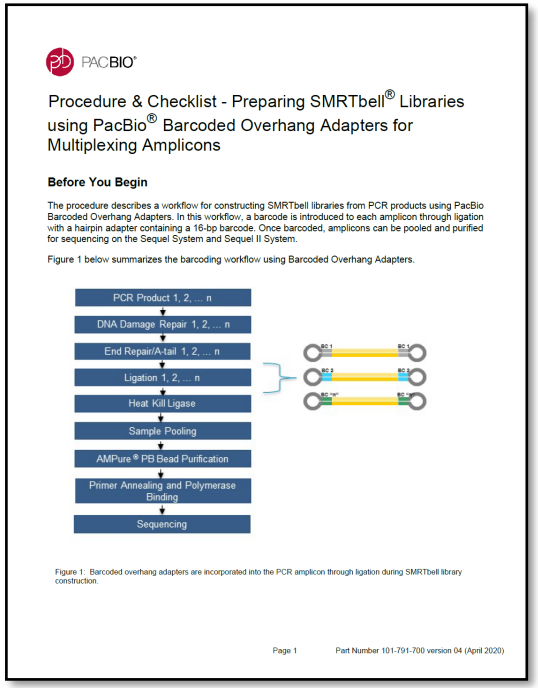
MULTIPLEXED AMPLICON SEQUENCING: HOW TO GET STARTED

Application-Specific Best Practices Guide → **Application-Specific Procedure & Checklist** → **Application Consumable Bundle Purchasing Guide** → **Library Construction, Sequencing & Analysis**



[Application Brief: Targeted sequencing for amplicons – Best Practices](#) (BP105-071919)

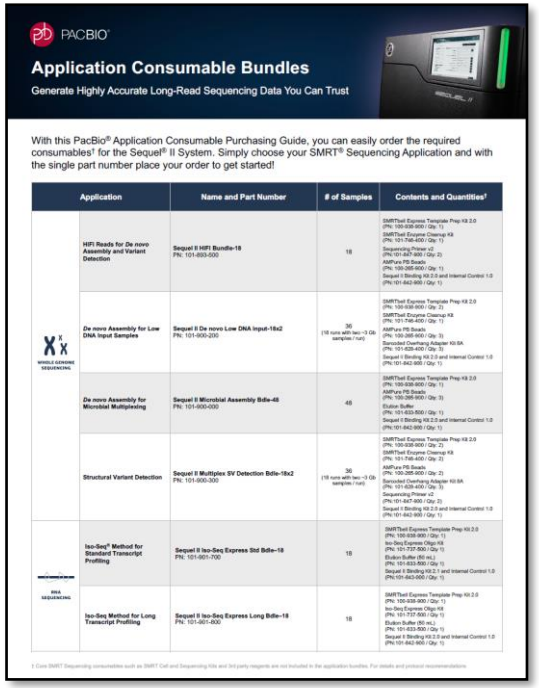
Summary overview of application-specific sample preparation and data analysis workflow recommendations



[Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons](#) (101-791-700)

[Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing](#) (101-791-800)

[Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing](#) (101-921-300)



[PacBio Application Consumable Bundle Purchasing Guide](#) (PG100-082620)

Purchasing Guide enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II and IIe Systems*

PCR Amplicon Generation

Barcoding Options:
Barcoded Target-Specific Primers or Barcoded Universal Primers (BUP)

Library Construction (SMRTbell Express TPK 2.0)

Barcoding Options:
Barcoded Overhang Adapters (BOA)

HiFi Sequencing

Generate Up To 3 Million HiFi Reads per SMRT Cell 8M

Data Analysis (SMRT Link)

CCS Analysis
Demultiplexing Analysis

* Application Consumable Bundles include reagents for library construction, primer annealing and polymerase binding. Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles

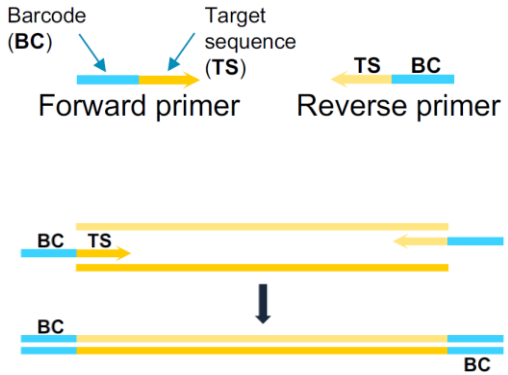
Multiplexed Amplicon Library Sample Preparation Workflow Overview

BARCODING OPTIONS FOR MULTIPLEXED AMPLICON SEQUENCING

Barcoded Target-Specific Primers

Customer-Supplied Primers

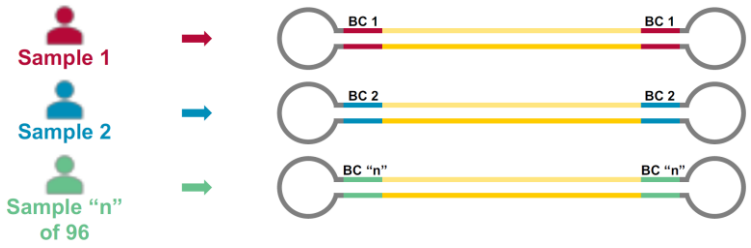
- Target-specific primers are tailed with PacBio 16-bp barcodes to produce **symmetrically** *or* **asymmetrically** barcoded samples using a 1-step PCR method.
- Can be used for small or large projects when validation of barcoded target-specific primers will be performed at the start of a project



Barcoded Overhang Adapters (BOA)

PacBio-Supplied BOAs or **Customer-Supplied BOAs**

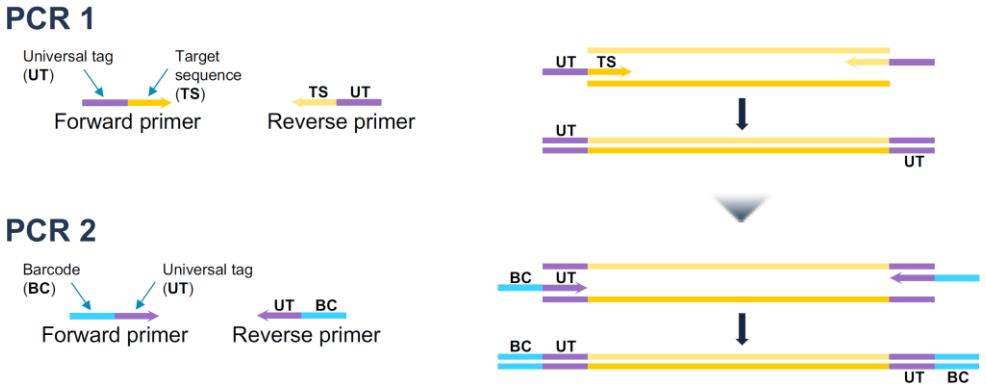
- PacBio 16-bp barcodes are added to amplicons through ligation of barcoded overhang SMRTbell adapters to produce **symmetrically** barcoded samples. (Procedure: PN 101-791-700)
- Two sets of 8 barcoded overhang adapters are commercially available from PacBio (PN 101-628-400/500)
- Recommended for smaller projects (≤96 samples) using validated PCR systems and off-the-shelf assays.



Barcoded Universal Primers (BUP)

PacBio-Supplied Barcoded Universal Primers or **Customer-Supplied Barcoded Universal Primers (e.g., Barcoded M13 Primers)**

- PacBio 16-bp barcodes are added to amplicons through a 2-step PCR method using Universal Sequence-tagged target-specific primers and Universal Sequence-tagged 16-bp barcoded primers. (Procedure: PN 101-791-800 / 101-921-300)
- For **symmetric** barcoding of ≤96 amplicon samples, we recommend using our 96 Barcoded Universal Primers kit (PN 101-629-100)
- For **asymmetric** barcoding of >96 amplicon samples, we recommend using the Barcoded M13 Primer approach with custom-ordered barcoded M13 primers (see **Appendix 1**).



https://www.pacb.com/products-and-services/consumables/multiplexing-kits/
https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/

WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO-BARCODED TARGET-SPECIFIC PRIMERS

Barcoded amplicons are generated *via* a **1-step PCR**, Once the PCR products are barcoded, they can be pooled into a ***single*** tube for SMRTbell library construction.

1-Step PCR Amplicon Generation with Customer-Supplied Target-Specific Primers

- Perform PCR using Target-Specific Primers tailed with PacBio 16-bp barcode sequences
- Minimum required input DNA mass per pooled sample for library construction is ≥250 ng (dependent on insert size)

SMRTbell Library Construction (4 hrs)

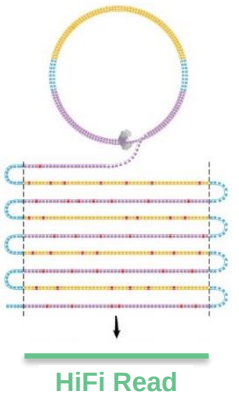
- Pool barcoded amplicon samples as described in the 'Sample Pooling' step on page 7 of the ***Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplexing Amplicons*** (PN 101-791-800) and proceed with library construction

HiFi Sequencing

- Follow ***Quick Reference Cards*** for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

Data Analysis

- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools



PacBio HiFi reads achieve >99.9% accuracy

* *Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO-BARCODED OVERHANG ADAPTERS (BOA)

A barcode is introduced to each amplicon *via* **ligation with a hairpin adapter** containing a 16-bp PacBio barcode sequence. Once barcoded, amplicons can be pooled as a ***single*** SMRTbell library sample for sequencing.

Input PCR Amplicon QC

- Minimum required input DNA mass per amplicon for library construction is 10 ng

SMRTbell Library Construction (<5 hrs)

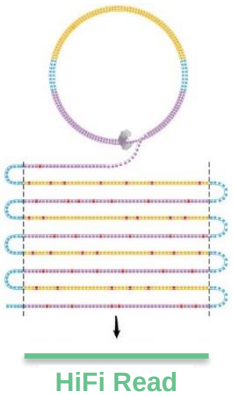
- Follow ***Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons*** (PN 101-791-700) and pool samples
- Use PacBio Barcoded Overhang Adapter Kit 8A//8B (PN 101-628-400/500) to multiplex up to 16 samples
- If multiplexing **>16** samples, order barcoded overhang adapters directly from a third-party oligo synthesis provider

HiFi Sequencing

- Follow ***Quick Reference Cards*** for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

Data Analysis

- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools



PacBio HiFi reads achieve >99.9% accuracy

** Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO-BARCODED UNIVERSAL PRIMERS (BUP) OR BARCODED M13 PRIMERS

Barcoded amplicons are generated *via* a **2-step PCR**, Once the PCR products are barcoded, they can be pooled into a ***single*** tube for SMRTbell library construction.

2-Step PCR Amplicon Generation with Barcoded Universal Primers or Barcoded M13 Primers

- Follow ***Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplexing Amplicons*** (PN 101-791-800)
- Or follow ***Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing*** (PN 101-921-300)

SMRTbell Library Construction (4 hrs)

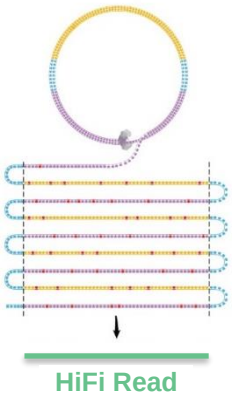
- Pool barcoded amplicon samples and proceed with library construction
- Minimum required input DNA mass per pooled sample for library construction is ≥250 ng

HiFi Sequencing

- Follow ***Quick Reference Cards*** for primer annealing, polymerase binding, complex cleanup and sample loading
- Generate up to 3 million HiFi reads per SMRT Cell 8M* (dependent on insert size)

Data Analysis

- Demultiplex barcodes using SMRT Link
- Analyze HiFi data using SMRT Link or other third-party tools



PacBio HiFi reads achieve >99.9% accuracy

** Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

Amplicon Sample QC Requirements and SMRTbell Express TPK 2.0 Handling Recommendations

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 in the following sections for generating **high-quality amplicons** suitable for SMRTbell library preparation and sequencing.

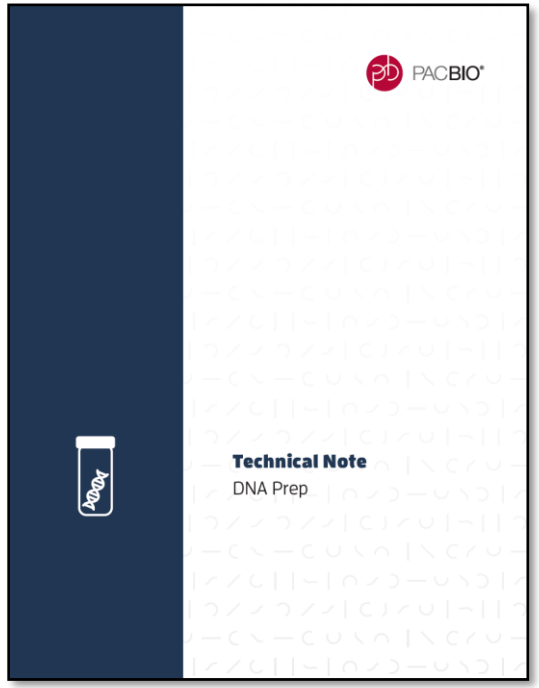
BEST PRACTICES FOR GENERATING HIGH-QUALITY PCR AMPLICONS (CONT.)

1. **Begin with high-quality nucleic acids and work in a clean environment.**
   a. For targeted sequencing of genomic DNA (gDNA) samples, starting with **high-quality DNA** will result in better performance during sequencing.
      - Technical Note: DNA Prep (PN TN101-061920) 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

   b. If extracted nucleic acids must be stored, **freeze at high concentrations** in appropriately-buffered solutions.

   c. To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, **sub-aliquot DNA into smaller volumes for storage**.

   d. 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**.



BEST PRACTICES FOR GENERATING HIGH-QUALITY PCR AMPLICONS (CONT.)

2. **Use PCR reagents and conditions for generating target-specific, full length amplicons.**
    a. Use the highest-fidelity polymerase compatible with your PCR amplification system.
    b. Use desalted or HPLC-purified oligo primers; damaged bases at the ends of the amplicons cannot be repaired by DNA Damage Repair enzymes.
    c. Optimize PCR conditions to minimize total time spent at high (>65°C) temperatures, particularly during denaturation.
    d. PCR extension time should be long enough to ensure complete extension, taking into consideration the polymerase used and target amplicon size.
        a. For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps.
        b. 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

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 short-amplicon samples (<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

https://www.thermofisher.com/order/catalog/product/Q33230#/Q33230

EVALUATION OF PCR AMPLICON DNA PURITY

- Amplicon DNA purity can be assessed through UV-spectrophotometry using a Nanodrop spectrophotometer
- For pure DNA, A260/280 ratio is typically **~1.8** or higher and A260/230 ratio is **≥2.0** or higher
- For samples with ratios that fall outside the expected optimal values, PacBio recommends performing an AMPure bead purification step followed by re-assessment of quantity and purity of the DNA sample
  - See Technical Note: DNA Prep (PN TN101-061920) for recommended DNA cleanup procedure

260/280 Ratio

- A low A260/A280 ratio may indicate the presence of protein, phenol, or 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 normally indicative of an issue.

260/230 Ratio

- A low A260/A230 ratio may be the result of:
  - 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.

PacBio recommends only proceeding with DNA samples that have an absorbance **A260/A280 ratio between 1.8 – 2.0** and a **A260/A230 ratio between 2.0 – 2.5**.

NanoDrop 2000 System (Thermo Fisher Scientific)

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 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

PacBio recommends pooling amplicons of **similar size** (within approx. +/- 15% of the mean size) for sequencing on a single SMRT Cell

Bioanalyzer 2100 System (Agilent Technologies)

Fragment Analyzer System (Agilent Technologies)

Femto Pulse System (Agilent Technologies)

RECOMMENDATIONS FOR AMPURE PB BEAD PURIFICATION OF AMPLICON SAMPLES

- It is highly recommended to purify amplicons **before** SMRTbell library construction to remove PCR reagents, buffers, primer dimers or short non-specific PCR products.
- Depending on the amplicon size, the required concentration of AMPure PB beads for purification varies.
- Use the table below to determine the appropriate concentration of AMPure PB beads to use for your specific sample.

AMPURE PB BEAD RECOMMENDATIONS BASED ON AMPLICON SIZE.

AMPLICON SIZE	AMPURE PB BEAD RATIO
250 – 500 bp	1.8X
500 – 1000 bp	1.0X
1 – 3 kb	0.6X
3 – 10 kb	0.45X
15 kb	0.45X

SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 REAGENT HANDLING RECOMMENDATIONS

- Several reagents in the kit are sensitive to temperature and vortexing
- PacBio highly recommends:
  - Never leaving reagents at room temperature
  - Working on ice at all times when preparing master mixes
  - Finger tapping followed by a quick-spin prior to use

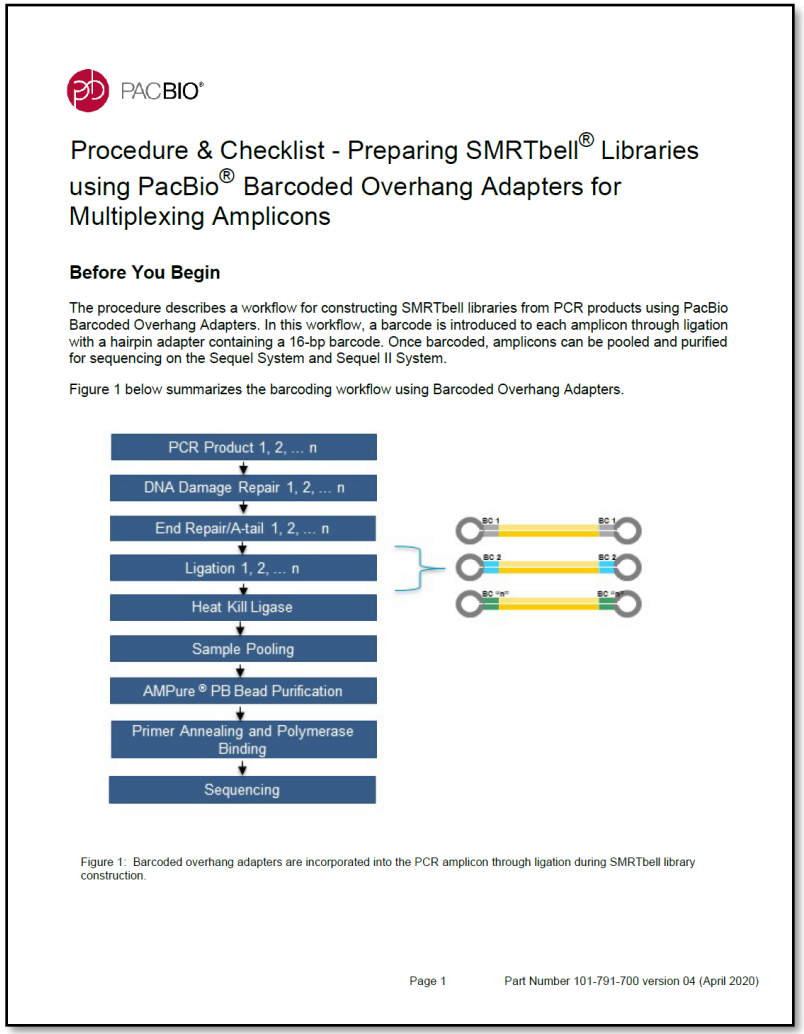
LIST OF TEMPERATURE-SENSITIVE REAGENTS INCLUDED IN SMRTBELL EXPRESS TPK 2.0.

REAGENT	WHERE USED
DNA Damage Repair Mix v2	DNA Damage Repair
End Prep Mix	End-Repair/A-tailing
Overhang Adapter v3	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation

Multiplexed Amplicon Library Preparation Using PacBio Barcoded Overhang Adapters (BOA)

PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED OVERHANG ADAPTERS FOR MULTIPLEXING AMPLICONS

- This protocol (PN 101-791-700) describes a workflow for constructing symmetrically-barcoded SMRTbell libraries from up to 96 PCR amplicon samples using **PacBio Barcoded Overhang Adapters (BOA)** for multiplexed sequencing on the Sequel, Sequel II and Sequel IIe System (Sequel Systems)
- Protocol document contains:
  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing and input PCR amplicon QC
  2. General recommendations for planning multiplexing experiments using Barcoded Overhang Adapters
  3. Instructions for constructing **symmetrically barcoded SMRTbell libraries** with input PCR amplicons using PacBio's SMRTbell Express Template Prep Kit 2.0 and PacBio Barcoded Overhang Adapter Kit 8A/8B (PN 101-628-400/500) or customer-supplied barcoded overhang adapters*
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel Systems
- For additional guidance on preparing HLA amplicon samples for multiplexed SMRT Sequencing, see ***Reference Guide – Human Leukocyte Antigen (HLA) Sequencing*** (PN 102-066-200) and **Appendix 2** of this presentation



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Procedure & Checklist - Preparing SMRTbell® Libraries using PacBio® Barcoded Overhang Adapters for Multiplexing Amplicons

Before You Begin

The procedure describes a workflow for constructing SMRTbell libraries from PCR products using PacBio Barcoded Overhang Adapters. In this workflow, a barcode is introduced to each amplicon through ligation with a hairpin adapter containing a 16-bp barcode. Once barcoded, amplicons can be pooled and purified for sequencing on the Sequel System and Sequel II System.

Figure 1 below summarizes the barcoding workflow using Barcoded Overhang Adapters.

Figure 1: Barcoded overhang adapters are incorporated into the PCR amplicon through ligation during SMRTbell library construction.

Page 1 Part Number 101-791-700 version 04 (April 2020)

* Barcoded Overhang Adapter Kits 8A & 8B support multiplexing of up to 16 samples. If multiplexing projects require more than 16 samples (up to 96 samples), we offer a list of barcoded overhang adapter sequences that you can order from an oligo synthesis provider.

https://www.pacb.com/support/documentation/

REQUIRED MATERIALS & EQUIPMENT FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING BARCODED OVERHANG ADAPTERS

ITEM	WHERE USED	VENDOR	PART NUMBER
Barcoded Overhang Adapters	Library Prep	PacBio or Third-Party Oligo Vendor	See 'Barcoded Overhang Adapters' note below
SMRTbell Express Template Prep Kit 2.0	Library Prep	PacBio	100-938-900
AMPure PB Bead Kit	Purification	PacBio	100-265-900
8-Channel or 12-Channel Pipette	High-Throughput Pipetting	Any	Vendor-specific
PCR 8-Tube Strips	Tubes for reactions	Any	Vendor-specific
96-Well Plate	Tubes for reactions	Any	Vendor-specific

Barcoded Overhang Adapters

- 16 Barcoded Overhang Adapters are commercially available from PacBio:
  - PacBio Barcoded Overhang Adapter Kit 8A (PN 101-628-400) and PacBio Barcoded Overhang Adapter Kit 8B (PN 101-628-500). We recommend using both sets for multiplexing up to 16 samples
- **If multiplexing projects require more than 16 samples**, PacBio provides a list of 96 barcoded overhang adapter (BOA) sequences that you can order from an oligo synthesis provider (see our Multiplexing Resources website)
  - List contains BOA sequence information (5' → 3') along with their recommended synthesis scale and purification method
  - Annealing procedure for forming SMRTbell hairpins is described in the "Annealing PacBio SMRTbell Overhang Adapters" section of this procedure

MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO BARCODED OVERHANG ADAPTERS: DETAILED WORKFLOW OVERVIEW

1. Input PCR Amplicon QC

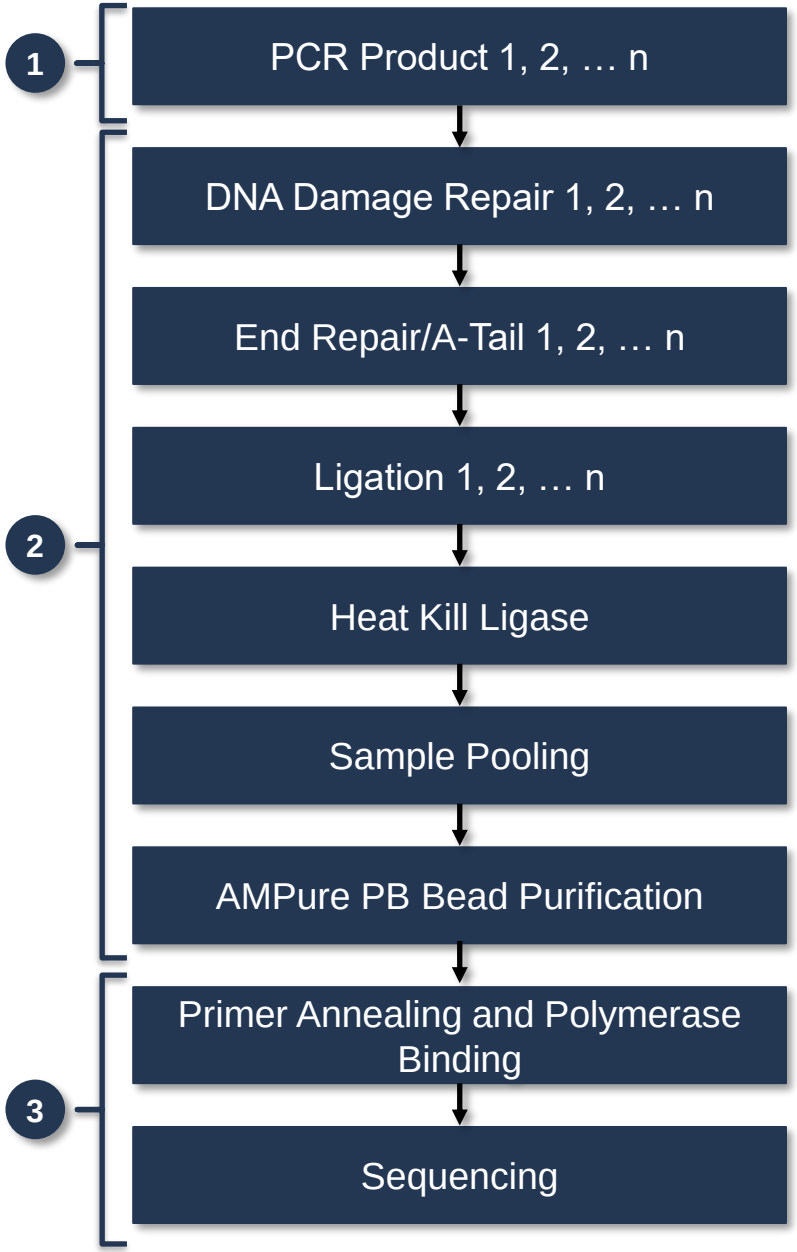
- Verify input amplicon sample concentration, purity and size distribution
- Minimum required input mass per amplicon for library construction depends on amplicon size:
  - E.g., 10 ng for 150 bp amplicon to 150 ng for 15,000 bp amplicon

2. Barcoded SMRTbell Express TPK 2.0 Library Construction

- Construct barcoded SMRTbell libraries using PacBio Barcoded Overhang Adapters
- Typical library yield ≥40%
- <5 hours hands-on time (depends on multiplex design)

3. Sequencing Preparation

- Anneal Sequencing Primer, bind Polymerase, perform Complex Cleanup
- For specific sample setup and run design guidance, refer to Loading and Pre-Extension Recommendations Quick Reference Cards for Sequel, Sequel II and Sequel IIe Systems



4. Data Analysis

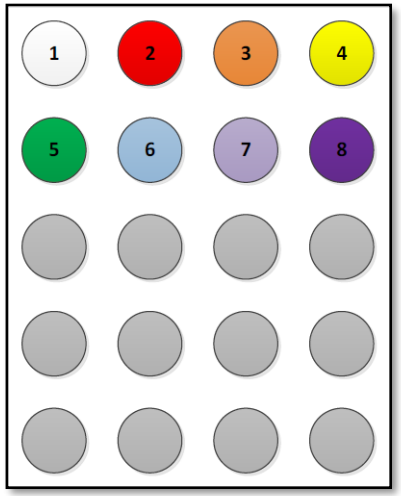
- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- De-multiplex barcodes within SMRT Link GUI or on the command line
- Amplicon data analysis using SMRT Analysis Applications (CCS, Minor Variants Analysis)
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools

- PacBio highly recommends **SMRT Link v9.0 or higher** to perform demultiplexing of your amplicon sequencing data
- SMRT Link GUI supports demultiplexing of up to 10,000 barcoded samples per data set; support for >10,000 barcoded samples is available using the command-line interface

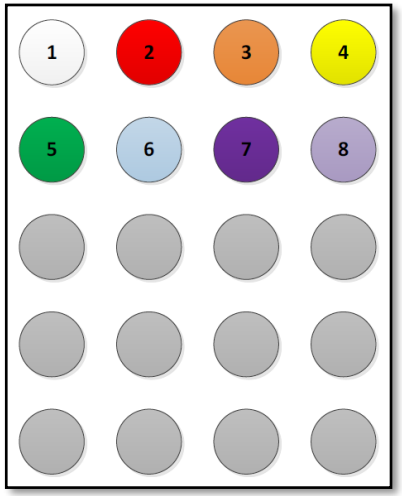
PACBIO BARCODED OVERHANG ADAPTER KIT 8A / 8B IS AVAILABLE FOR MULTIPLEXING UP TO 16 POOLED AMPLICON SAMPLES

- 16 Barcoded overhang adapters are commercially available from PacBio:
  - PacBio Barcoded Overhang Adapter Kit 8A, PN 101-628-400; and
  - PacBio Barcoded Overhang Adapter Kit 8B, PN 101-628-500)
- Each Barcoded Overhang Adapter (BOA) Kit 8A or 8B contains sufficient reagents to perform 12 ligation reactions for each BOA to support multiplexed amplicon sequencing
- Each PacBio barcode sequence is 16 bp in length
- We recommend using both BOA kits (8A and 8B) for multiplexing up to 16 samples

If multiplexing projects require **>16** samples, PacBio's Multiplexing Resources website provides a **list** of **96 barcoded overhang adapter (BOA)** sequences that you can order from a third-party oligo synthesis provider



Barcoded Overhang Adapter Kit - 8A (PN 101-628-400)	
Tube #	**Description**
1	TUBE, Bar Over Adapt - bc1001
2	TUBE, Bar Over Adapt - bc1002
3	TUBE, Bar Over Adapt - bc1003
4	TUBE, Bar Over Adapt - bc1008
5	TUBE, Bar Over Adapt - bc1009
6	TUBE, Bar Over Adapt - bc1010
7	TUBE, Bar Over Adapt - bc1011
8	TUBE, Bar Over Adapt - bc1012



Barcoded Overhang Adapter Kit - 8B (PN 101-628-500)	
Tube #	**Description**
1	TUBE, Bar Over Adapt - bc1015
2	TUBE, Bar Over Adapt - bc1016
3	TUBE, Bar Over Adapt - bc1017
4	TUBE, Bar Over Adapt - bc1018
5	TUBE, Bar Over Adapt - bc1019
6	TUBE, Bar Over Adapt - bc1020
7	TUBE, Bar Over Adapt - bc1021
8	TUBE, Bar Over Adapt - bc1022

PacBio barcode sequence FASTA files for Sequel Systems can be obtained here: https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/

GENERAL RECOMMENDATIONS FOR PLANNING MULTIPLEXING EXPERIMENTS USING PACBIO BARCODED OVERHANG ADAPTERS

1. Review the section on Page 3 of the procedure that describes '**Best Practices for Generating High-Quality PCR Products for PacBio Sequencing**.'

2. Accurately assess the sizes of the amplicons that are being multiplexed. **Pool amplicons of similar size** (within +/- 15% of the mean size).

3. Determine the number of different amplicon samples to be multiplexed.

   - We recommend initially starting with a low number of amplicon samples to multiplex. As you become more experienced with the workflow and understand the sequencing coverage performance of your amplicons, you may decide to increase the level of multiplexing.

4. Estimate the input DNA mass requirement per amplicon for library construction.

   - See '**Estimating Input Requirements for Library Construction**' section on Page 3 of the procedure.

5. Determine the appropriate concentration of AMPure PB beads to use for the purification steps.

   - See '**Recommendations for AMPure PB Bead Purifications**' on Page 4 of the procedure.

ESTIMATING INPUT AMPLICON MASS REQUIREMENTS FOR MULTIPLEXED SMRTBELL LIBRARY CONSTRUCTION USING PACBIO BARCODED OVERHANG ADAPTERS

Refer to **Table 2** on Page 3 of the procedure for DNA input requirements for SMRTbell library construction

INPUT DNA MASS REQUIRED PER AMPLICON FOR MULTIPLEXING USING BARCODED OVERHANG ADAPTERS (BOA). (SEE "POOLING" SECTION ON PAGE 9 OF THE PROCEDURE).

AMPLICON SIZE (BP)	MINIMUM INPUT PER AMPLICON INTO DNA DAMAGE REPAIR (ng)	TARGET DNA MASS (ng) AFTER POOLING*
250 – 500	10 ng per amplicon for ≥25-plex	250
501 – 1000	10 ng per amplicon for ≥25-plex	250
1001 – 3000	50 ng per amplicon for ≥10-plex	500
3001 – 10000	100 ng per amplicon for ≥10-plex	1000
10001 - 15000	150 ng per amplicon for ≥10-plex	1500

* The value in the "Target DNA Mass (ng) After Pooling" column is the recommended minimum amount of SMRTbell library after pooling of BOA ligation products.

- The numbers in the "Minimum Input per Amplicon into DNA Damage Repair" column are the recommended **per-amplicon masses** required for the first enzymatic reaction step in library construction (DNA Damage Repair), regardless of the number of amplicons to be multiplexed.

POOLING BOA AMPLICON SAMPLES AFTER SMRTBELL LIBRARY CONSTRUCTION

After the ligation (+ heat kill) step with the barcoded overhang adapters, amplicon samples can be pooled together for AMPure PB bead purification.

- Ensure that the pooled sample contains the minimum required total mass (ng) of SMRTbell library as indicated in **Table 2** ('Target DNA Mass (ng) After Pooling' column)

Heat kill of ligase *prior* to pooling is necessary in order to prevent unwanted further ligation of different combinations of residual barcoded overhang adapter hairpins to any open-ended SMRTbell templates present in the reaction tube.

1. After performing the ligase heat kill, pool all reactions (~16.3 µL each) into a **single** 1.5- or 2.0-mL DNA Lo-Bind tube.
2. Mix and spin down the contents of the tube with a quick spin in a microfuge.
3. Proceed to the next step to purify the pooled library with AMPure PB beads.

USE A MULTI-CHANNEL PIPETTE FOR HIGH-THROUGHPUT AMPLICON SEQUENCING APPLICATIONS

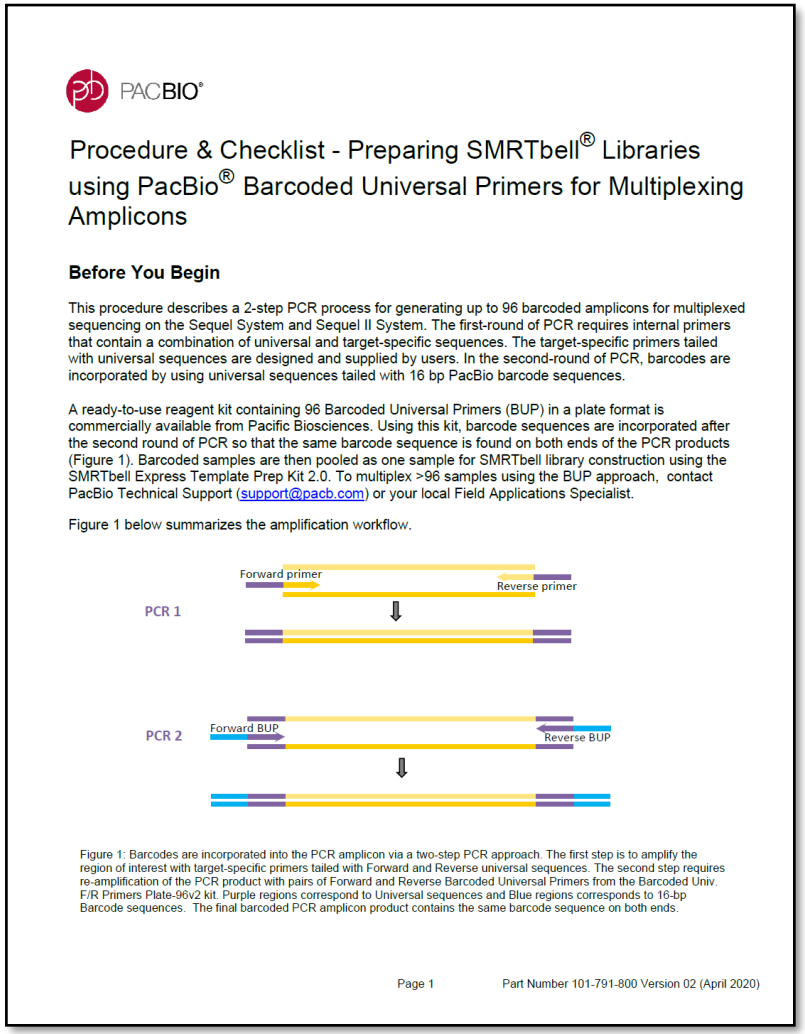
When working with a large number of reactions, PacBio highly recommends using a multichannel pipette to transfer small aliquots of master mixes to a 96-well PCR plate or PCR tubes

1. Prepare master mixes according to the instructions in the DNA Damage Repair, End-Repair/A-tailing and Adapter Ligation sections of this procedure, or use the Express Amplicon Master Mix Calculator.
2. Transfer aliquots of the master mix into an 8-tube strip using a single channel pipette (1/8th master mix volume to each of the eight well of the strip tube). Each tube can accommodate up to 200 µL of liquid.
3. Using an 8-channel pipette, transfer the required reaction volume of the master mix from the 8-tube strip into the appropriate sample wells of a 96-well plate.
4. Repeat until all required reaction wells in the sample plate are filled.

Multiplexed Amplicon Library Preparation Using PacBio Barcoded Universal Primers (BUP)

PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED UNIVERSAL PRIMERS FOR MULTIPLEXING AMPLICONS

- This protocol (PN 101-791-800) describes a 2-step PCR method for generating up to 96 symmetrically barcoded amplicon products using the **PacBio Barcoded Universal Primers (BUP)** kit (PN 101-629-100) and performing SMRTbell library construction for multiplexed BUP amplicon sequencing on Sequel, Sequel II and Sequel IIe Systems
- Protocol document contains:
  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing
  2. Instructions for generating **symmetrically barcoded amplicons** using a 2-Step PCR method with PacBio's Barcoded Universal Primer Kit* and performing equimolar pooling of barcoded amplicon products
  3. Instructions for constructing SMRTbell libraries with pooled barcoded PCR amplicons using PacBio's SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel, Sequel and Sequel IIe Systems
- For guidance on generating **asymmetrically** barcoded amplicon samples for higher multiplexing (>96-plex) using customer-supplied barcoded M13 primers, see ***Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing*** (PN 101-921-300) and **Appendix 1** of this presentation
- For guidance on preparing HLA amplicon samples for multiplexed SMRT Sequencing, see ***Reference Guide – Human Leukocyte Antigen (HLA) Sequencing*** (PN 102-066-200) and **Appendix 2** of this presentation



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Procedure & Checklist - Preparing SMRTbell® Libraries using PacBio® Barcoded Universal Primers for Multiplexing Amplicons

Before You Begin

This procedure describes a 2-step PCR process for generating up to 96 barcoded amplicons for multiplexed sequencing on the Sequel System and Sequel II System. The first-round of PCR requires internal primers that contain a combination of universal and target-specific sequences. The target-specific primers tailed with universal sequences are designed and supplied by users. In the second-round of PCR, barcodes are incorporated by using universal sequences tailed with 16 bp PacBio barcode sequences.

A ready-to-use reagent kit containing 96 Barcoded Universal Primers (BUP) in a plate format is commercially available from Pacific Biosciences. Using this kit, barcode sequences are incorporated after the second round of PCR so that the same barcode sequence is found on both ends of the PCR products (Figure 1). Barcoded samples are then pooled as one sample for SMRTbell library construction using the SMRTbell Express Template Prep Kit 2.0. To multiplex >96 samples using the BUP approach, contact PacBio Technical Support (support@pacb.com) or your local Field Applications Specialist.

Figure 1 below summarizes the amplification workflow.

Figure 1: Barcodes are incorporated into the PCR amplicon via a two-step PCR approach. The first step is to amplify the region of interest with target-specific primers tailed with Forward and Reverse universal sequences. The second step requires re-amplification of the PCR product with pairs of Forward and Reverse Barcoded Universal Primers from the Barcoded Univ. F/R Primers Plate-96v2 kit. Purple regions correspond to Universal sequences and Blue regions corresponds to 16-bp Barcode sequences. The final barcoded PCR amplicon product contains the same barcode sequence on both ends.

Page 1 Part Number 101-791-800 Version 02 (April 2020)

https://www.pacb.com/support/documentation/

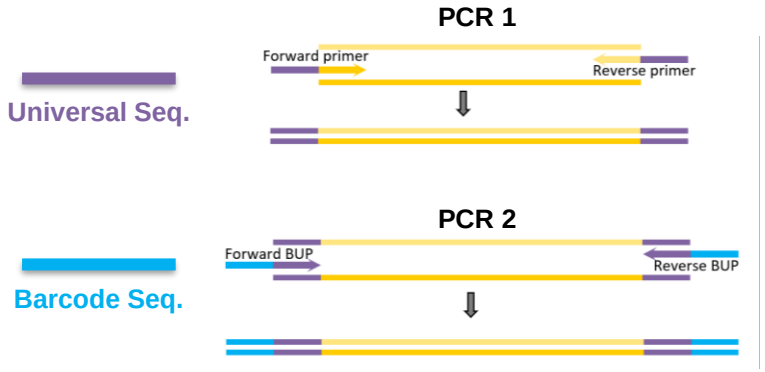
REQUIRED MATERIALS & EQUIPMENT FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING BARCODED UNIVERSAL PRIMERS

ITEM	WHERE USED	VENDOR	PART NUMBER
Target Specific Primers tailed with F/R Universal Sequences (Customer-supplied)	PCR Amplification (1st-Round)	Oligo Synthesis Company	N/A
Barcoded Universal F/R Primers Plate-96v2	PCR Amplification (2nd-Round)	PacBio	101-629-100
High Fidelity DNA Polymerase Master Mix (Two recommended mixes are listed below)			
Phusion High-Fidelity PCR Master Mix with HF Buffer; or	PCR Amplification	Thermo Scientific	F531S
KAPA HiFi HotStart ReadyMix PCR Kit	PCR Amplification	Roche	KK2600, KK2601, or KK2602
SMRTbell Express Template Prep Kit 2.0	Library Prep	PacBio	100-938-900
AMPure PB Bead Kit	Purification	PacBio	100-265-900
Multichannel Pipettor	High-Throughput Pipetting	Any	Vendor-specific

MULTIPLEXED AMPLICON LIBRARY PREPARATION USING PACBIO BARCODED UNIVERSAL PRIMERS: DETAILED WORKFLOW OVERVIEW

1. 2-Step PCR Amplicon Generation with Barcoded Universal Primer Kit (PN 101-629-100)

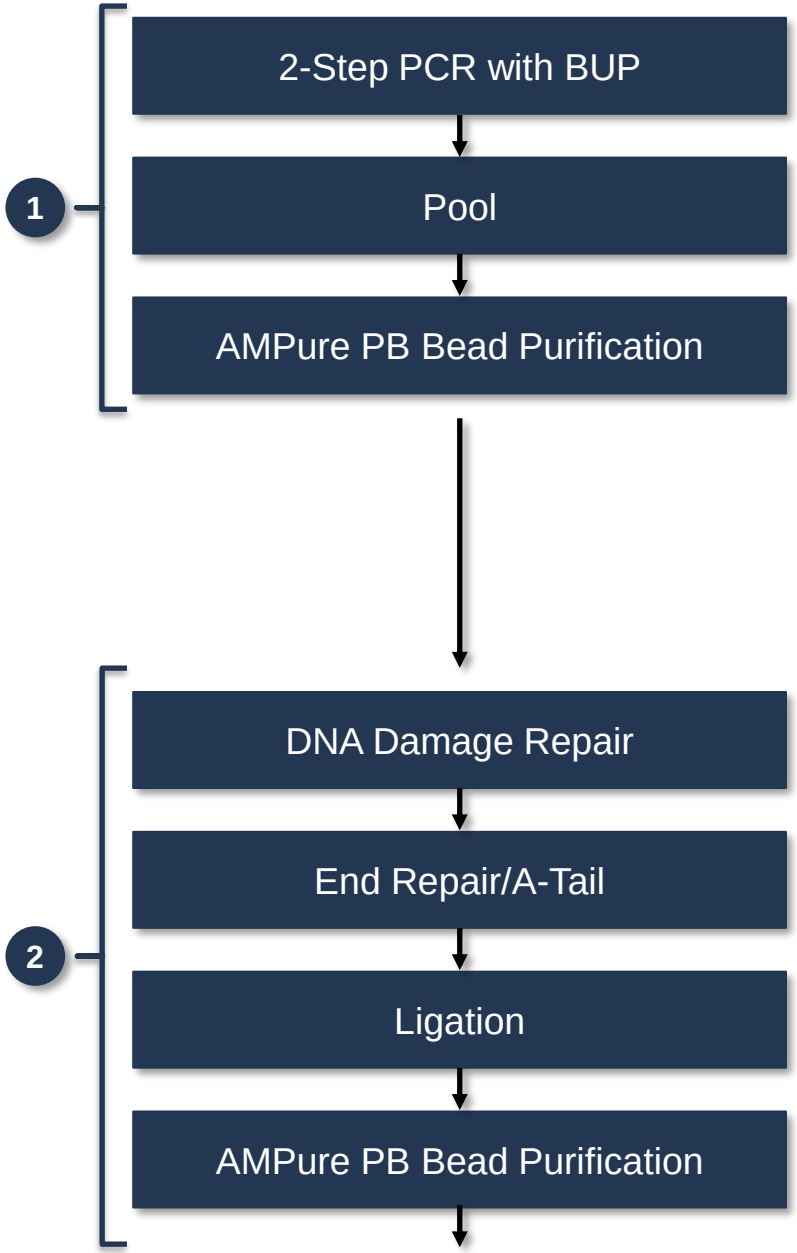
- Perform 1st-round PCR using Target Specific Primers tailed with Universal Sequences (US)
- Perform 2nd-round PCR Using Barcoded Universal Primers (BUP)



- Perform equimolar pooling of barcoded amplicon products
- Purify single, pooled amplicon sample using AMPure PB beads

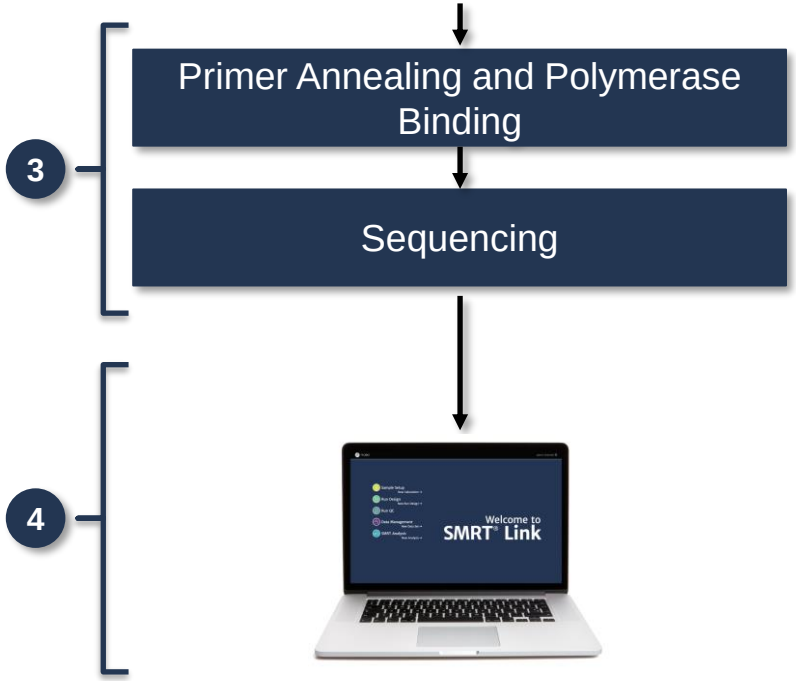
2. SMRTbell Express TPK 2.0 Library Construction

- Required input mass per pooled sample for library construction is ≥250 ng (depending on the insert size)
- Typical library yield ≥40%
- ~4 hours hands-on time



3. Sequencing Preparation

- Anneal Sequencing Primer, bind Polymerase, perform Complex Cleanup
- For specific sample setup and run design guidance, refer to Loading and Pre-Extension Recommendations Quick Reference Cards for Sequel, Sequel and Sequel IIe Systems



4. Data Analysis

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- De-multiplex barcodes within SMRT Link GUI or on the command line
- Amplicon data analysis using SMRT Analysis Applications (CCS, Minor Variants Analysis)
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools

- PacBio highly recommends **SMRT Link v9.0 or higher** to perform demultiplexing of your amplicon sequencing data
- SMRT Link GUI supports demultiplexing of up to 10,000 barcoded samples per data set; support for >10,000 barcoded samples is available using the command-line interface

DESIGNING TARGET-SPECIFIC PRIMERS TAILED WITH UNIVERSAL SEQUENCES FOR 1ST-ROUND PCR AMPLIFICATION

PacBio highly recommends complying with the following requirements below when ordering oligos from your oligo synthesis provider

1. The 5' end of the primer **must be blocked** (e.g., 5AmMC6) to prevent amplicons carried over from the first round of PCR from forming SMRTbell templates during library construction, which can reduce the yield of barcoded reads during sequencing.
2. Oligos can be **desalted**.
3. Use the following **primer format**:

PRIMER TYPE	UNIVERSAL SEQUENCE	TARGET-SPECIFIC PRIMER	PRIMER TO ORDER
Forward_Internal_PCR_Primer	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac	FOR_EXAMPLE1	/5AmMC6/ gcagtcgaacatgtagctgactcaggtcac FOR_EXAMPLE1
Reverse_Internal_PCR_Primer	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag	REV_EXAMPLE2	/5AmMC6/ tggatcacttgtgcaagcatcacatcgtag REV_EXAMPLE2

The procedure was optimized using high fidelity Phusion Hot Start II for PCR. KAPA HiFi HotStart ReadyMix may also be used. **PCR optimizations are highly recommended** before proceeding with the second-round PCR step

PACBIO BARCODED UNIVERSAL PRIMERS PLATE-96 KIT FOR 2ND-ROUND PCR AMPLIFICATION TO GENERATE SYMMETRICALLY BARCODED SAMPLES

- To perform this procedure, barcoded universal primers are required
- 96 barcoded universal (forward/reverse) primer pairs are commercially available from PacBio in a ready-to-use plate kit format:
  - Barcoded Universal F/R Primers Plate-96 Kit v2, PN 101-629-100
- Each well on the plate contains a primer pair consisting of forward and reverse primers having the **same** (16-bp) PacBio barcode sequence
- Each barcode is present in only one well for a total of 96 different barcodes on the plate. Each primer has a Universal Sequence tag on the 3' end.

Reagent kit quantities support a **single use** of each of the 96 primers pairs in the plate for SMRTbell library preparations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC1001	BC1009	BC1017	BC1025	BC1033	BC1041	BC1049	BC1057	BC1065	BC1073	BC1081	BC1089
B	BC1002	BC1010	BC1018	BC1026	BC1034	BC1042	BC1050	BC1058	BC1066	BC1074	BC1082	BC1090
C	BC1003	BC1011	BC1019	BC1027	BC1035	BC1043	BC1051	BC1059	BC1067	BC1075	BC1083	BC1091
D	BC1004	BC1012	BC1020	BC1028	BC1036	BC1044	BC1052	BC1060	BC1068	BC1076	BC1084	BC1092
E	BC1005	BC1013	BC1021	BC1029	BC1037	BC1045	BC1053	BC1061	BC1069	BC1077	BC1085	BC1093
F	BC1006	BC1014	BC1022	BC1030	BC1038	BC1046	BC1054	BC1062	BC1070	BC1078	BC1086	BC1094
G	BC1007	BC1015	BC1023	BC1031	BC1039	BC1047	BC1055	BC1063	BC1071	BC1079	BC1087	BC1095
H	BC1008	BC1016	BC1024	BC1032	BC1040	BC1048	BC1056	BC1064	BC1072	BC1080	BC1088	BC1096

Figure illustration of mapping between a specific well location and a unique PacBio barcode sequence on a 96-well plate in the Barcoded Universal F/R Primers Plate-96 Kit v2 (PN 101-629-100)

PacBio barcode sequence FASTA files for Sequel Systems can be obtained here:
https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/

GENERAL RECOMMENDATIONS FOR PLANNING MULTIPLEXING EXPERIMENTS USING PACBIO BARCODED UNIVERSAL PRIMERS

1. Review the section on Page 3 of the procedure that describes '**Best Practices for Generating High-Quality PCR Products for PacBio Sequencing**.'

2. Accurately assess the sizes of the amplicons that are being multiplexed.
   - Pool amplicons of **similar size** (within +/- 15% of the mean size).

3. Determine the number of different amplicon samples to be multiplexed.
   - We recommend initially starting with a low number of amplicon samples to multiplex. As you become more experienced with the workflow and understand the sequencing coverage performance of your amplicons, you may decide to increase the level of multiplexing.

4. Estimate the input DNA mass requirements per pooled amplicon sample for library construction. See '**DNA Input Requirements for Library Construction**' section on Page 5 of the procedure.

5. Determine the appropriate concentration of AMPure PB beads to use for the purification steps. See '**Recommendations for AMPure PB bead Purifications**' section on Page 5 of the procedure.

ESTIMATING INPUT DNA MASS REQUIREMENTS FOR MULTIPLEXED AMPLICON LIBRARY CONSTRUCTION USING PACBIO BARCODED UNIVERSAL PRIMERS

Refer to Table 4 on Page 5 of the procedure for DNA input requirements for library construction

INPUT DNA RECOMMENDATIONS BASED ON AMPLICON SIZE.

AMPLICON SIZE	REQUIRED INPUT DNA MASS PER AMPLICON POOL (ng)
250-500 bp	250 – 500*
500-1000 bp	250 – 500*
1-3 kb	500 - 1000
3-10 kb	1000 - 2000
15 kb	1500 - 3000

* If input DNA mass is greater than 500 ng for amplicon sizes ≤1 kb, use 2X Barcoded Overhang Adapter in the ligation reaction.

- Note that the input requirements shown in Table 4 refer to the **total** pooled mass of DNA, not the mass of individual members of the pool. Samples should be present at **equimolar concentrations after pooling** (see “Best Practices for Equimolar Pooling” on Page 6 of the procedure).
- If necessary, replicate PCR reactions should be set up to obtain the required amount of DNA product.
  - This approach also minimizes PCR sampling bias for samples containing heterogeneous templates.

BEST PRACTICES FOR EQUIMOLAR POOLING OF BUP AMPLICON SAMPLES FOR SMRTBELL LIBRARY CONSTRUCTION

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to leverage the throughput capacity of a single SMRT Cell.

1. Pooling is generally recommended for amplicons of **similar sizes** (within +/-15% of the mean size).
2. Ideally, amplicons should be **AMPure PB bead purified prior to multiplexing**. See "**Concentrate PCR Products Using AMPure PB Beads**" section of the procedure.
3. To obtain equal representation of each amplicon in the data, it is important to pool samples in **equimolar** amounts.
    - To do this, purify with AMPure PB beads and quantify each amplicon using an appropriate DNA sizing QC analysis tool (e.g., Agilent Bioanalyzer System, Agilent Fragment Analyzer System, or Agilent Femto Pulse System, etc.)

BEST PRACTICES FOR EQUIMOLAR POOLING OF BUP AMPLICON SAMPLES FOR SMRTBELL LIBRARY CONSTRUCTION

For studies targeting a single consensus sequence per sample, amplicons may be multiplexed to leverage the throughput capacity of a single SMRT Cell.

4. Remove **non-specific** PCR products (contaminating bands) prior to pooling. The presence of non-specific products in the pool will impact sequencing data yield.
    a. If amplicons contain secondary bands that are <1.5 kb, it may be possible to remove them from >3 kb amplicons using AMPure PB bead purification using an appropriate concentration of beads.
    b. If the contaminating bands are close in size to or are larger than the desired amplicon, or are greater than 1.5 kb (i.e., they cannot be removed by AMPure PB bead purification), size selection using an automated size selection tool or other gel-based method may be necessary
    c. If removal of contaminating bands is not possible, we recommend re-optimization of the amplification reaction using more stringent PCR conditions.
    d. Always determine the concentration of the amplicon **target band** or **peak only**, and use this value to the mass or volume of the amplicon sample to be used during pooling.
    e. If presence of contaminating bands is determined to be acceptable, you may choose to include the amplicon in the sample pool. In such cases, it may be necessary to **increase the relative input** amounts of such amplicons during pooling in order to achieve adequate sequencing data yields
    f. Always determine the concentration of the **target band** or **peak**.

BEST PRACTICES FOR EQUIMOLAR POOLING OF BUP AMPLICON SAMPLES FOR SMRTBELL LIBRARY CONSTRUCTION (CONT.)

5. For **higher multiplexing (>96-plex)**, purifying and quantifying each individual PCR product may be difficult or impractical. A QC method that may work well is to load samples on an agarose gel to view the PCR products prior to pooling. This QC method may work well if PCR conditions are fully optimized to generate clean specific PCR products consistently.
    a. PCR products that show the same band intensity on a gel may be pooled by volume or mass.
        - To do this, include control fragments of known concentrations when loading samples to perform agarose gel electrophoresis. The pooled samples must meet the minimum input requirements listed on Table 4.
    b. For samples that show weak signals on a gel, increase the volume or mass used during pooling.

USING A MULTI-CHANNEL PIPETTE FOR HIGH-THROUGHPUT AMPLICON SEQUENCING APPLICATIONS

When working with a large number of reactions, PacBio highly recommend using a multi-channel pipette to transfer small aliquots of master mixes to a 96-well PCR plate or PCR tubes

1. Prepare master mixes according to the instructions in the ‘First-Round PCR Using Target Specific Primers Tailed with a Universal Sequence’ and ‘A Second-Round PCR Using Barcoded Universal Primers’ sections of this procedure.
2. Transfer aliquots of the master mix into an 8-tube strip using a single channel pipette (1/8th master mix volume to each of the eight well of the strip tube). Each tube can accommodate up to 200 µL of liquid.
3. Using an 8-channel pipette, transfer the required reaction volume of the master mix from the 8-tube strip into the appropriate sample wells of a 96-well plate.
4. Repeat until all required reaction wells in the sample plate are filled.

Multiplexed Amplicon Library Sequencing Workflow Recommendations

SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL SYSTEM (CHEMISTRY 3.0)

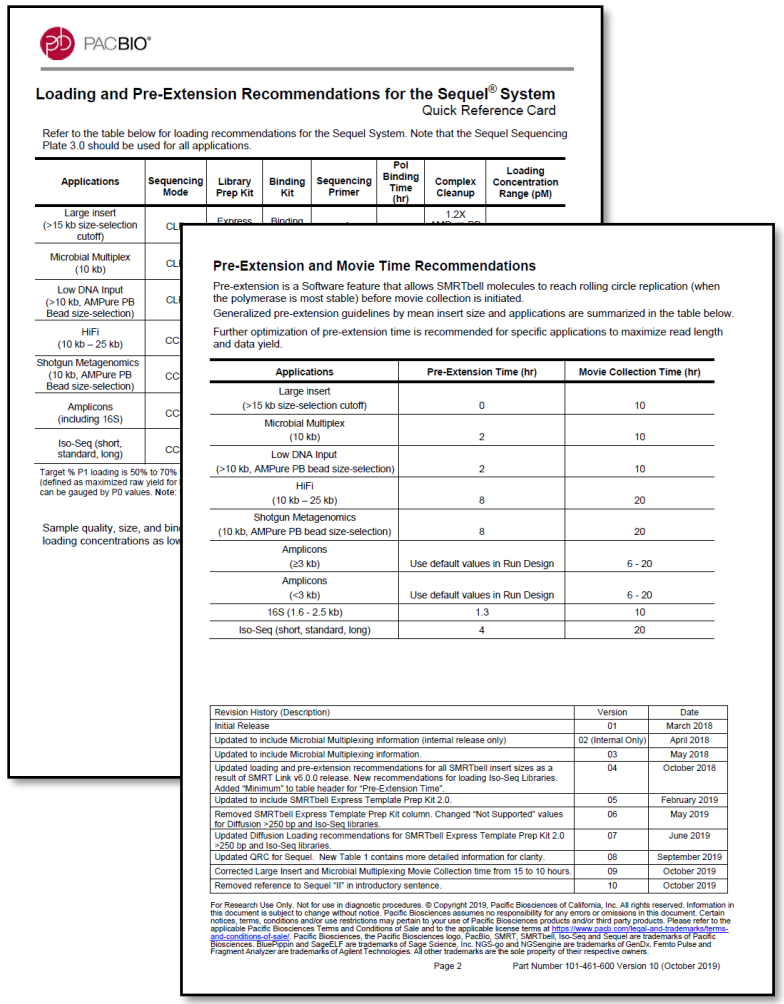
Sequel System Sample Setup Procedure for Amplicons **≥3 kb** and **<3 kb**

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel System for sequencing **amplicon samples ≥3 kb and <3 kb**

Applications	Sequencing Mode	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Amplicons (including 16S)	CCS	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	2 - 8

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Amplicons (≥3 kb)	Use default values in Run Design	6 - 20
Amplicons (<3 kb)	Use default values in Run Design	6 - 20

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ≥5 – 6 kb Amplicon Libraries: ~50%
  - ~3 – 4 kb Amplicon Libraries: ~40%
  - ~1 – 2 kb Amplicon Libraries: ~30%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Loading and Pre-Extension Recommendations for the Sequel® System
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel System. Note that the Sequel Sequencing Plate 3.0 should be used for all applications.

Applications	Sequencing Mode	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Large insert (>15 kb size-selection cutoff)	CL	Express	Binding			1.2X	
Microbial Multiplex (10 kb)	CL						
Low DNA Input (>10 kb, AMPure PB Bead size-selection)	CL						
HiFi (10 kb – 25 kb)	CC						
Shotgun Metagenomics (10 kb, AMPure PB Bead size-selection)	CC						
Amplicons (including 16S)	CC						
Iso-Seq (short, standard, long)	CC						

Target % P1 loading is 50% to 70% (defined as maximized raw yield for ... can be gauged by P0 values. Note:

Sample quality, size, and bin... loading concentrations as lo...

Pre-Extension and Movie Time Recommendations

Pre-extension is a Software feature that allows SMRTbell molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below.

Further optimization of pre-extension time is recommended for specific applications to maximize read length and data yield.

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Large insert (>15 kb size-selection cutoff)	0	10
Microbial Multiplex (10 kb)	2	10
Low DNA Input (>10 kb, AMPure PB bead size-selection)	2	10
HiFi (10 kb – 25 kb)	8	20
Shotgun Metagenomics (10 kb, AMPure PB bead size-selection)	8	20
Amplicons (≥3 kb)	Use default values in Run Design	6 - 20
Amplicons (<3 kb)	Use default values in Run Design	6 - 20
16S (1.6 - 2.5 kb)	1.3	10
Iso-Seq (short, standard, long)	4	20

Revision History (Description)	Version	Date
Initial Release	01	March 2018
Updated to include Microbial Multiplexing information (internal release only)	02 (Internal Only)	April 2018
Updated to include Microbial Multiplexing information.	03	May 2018
Updated loading and pre-extension recommendations for all SMRTbell insert sizes as a result of SMRT Link v6.0.0 release. New recommendations for loading Iso-Seq Libraries. Added "Minimum" to table header for "Pre-Extension Time".	04	October 2018
Updated to include SMRTbell Express Template Prep Kit 2.0.	05	February 2019
Removed SMRTbell Express Template Prep Kit column. Changed "Not Supported" values for Diffusion >250 bp and Iso-Seq libraries.	06	May 2019
Updated Diffusion Loading recommendations for SMRTbell Express Template Prep Kit 2.0 >250 bp and Iso-Seq libraries.	07	June 2019
Updated QRC for Sequel. New Table 1 contains more detailed information for clarity.	08	September 2019
Corrected Large Insert and Microbial Multiplexing Movie Collection time from 15 to 10 hours.	09	October 2019
Removed reference to Sequel "II" in introductory sentence.	10	October 2019

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.

SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL II AND IIe SYSTEMS (CHEMISTRY 2.0)

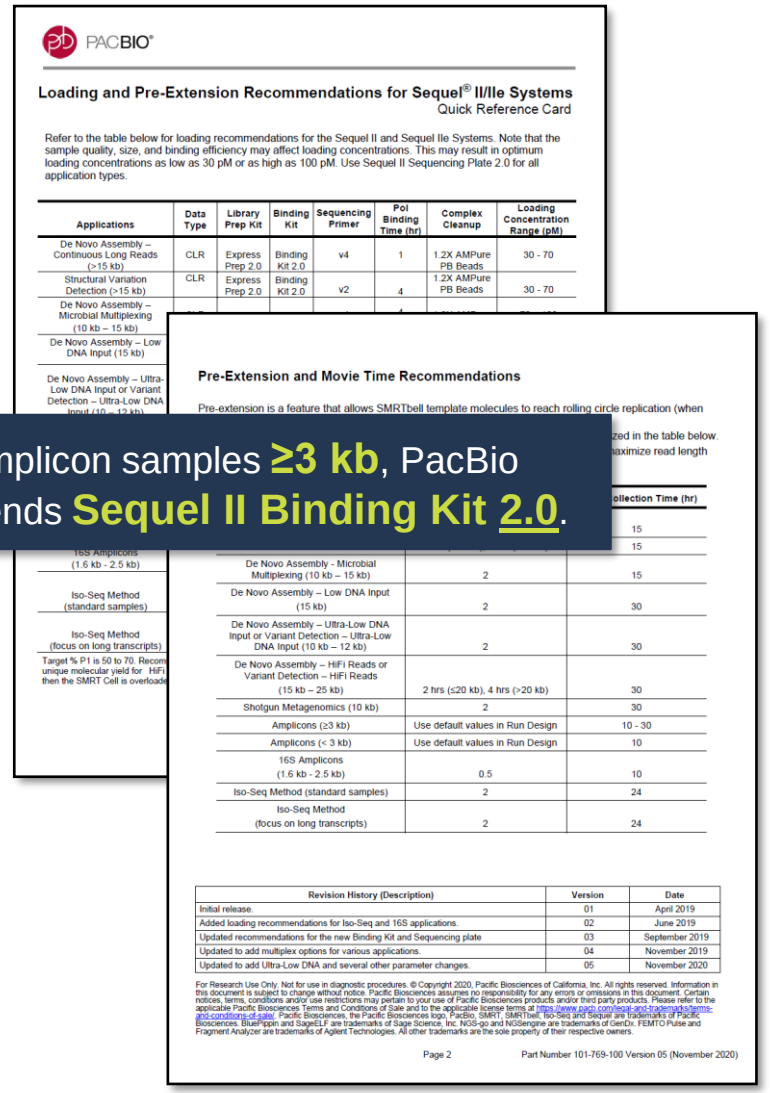
Sequel II and IIe System Sample Setup Procedure for Amplicons **≥3 kb**

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems for preparing ≥3 kb amplicon samples for sequencing
  - For **SMRT Link v10.0** (or higher): Select **'≥3 kb Amplicon Sequencing'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Amplicons (≥3 kb)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Amplicons (< 3 kb)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 150

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ≥5 – 6 kb Amplicon Libraries: ~50%
  - ~3 – 4 kb Amplicon Libraries: ~40%


PACBIO

Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 30 pM or as high as 100 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 - 70
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)							
De Novo Assembly – Low DNA Input (15 kb)							
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)							
16S Amplicons (1.6 kb - 2.5 kb)							
Iso-Seq Method (standard samples)							
Iso-Seq Method (focus on long transcripts)							
Target % P1 is 50 to 70. Recom... unique molecular yield for HiFi ... then the SMRT Cell is overloade...							

Pre-Extension and Movie Time Recommendations

Pre-extension is a feature that allows SMRTbell template molecules to reach rolling circle replication (when ...zed in the table below. ...aximize read length

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	15
De Novo Assembly – Low DNA Input (15 kb)	2	30
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	30
De Novo Assembly – HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	2 hrs (≤20 kb), 4 hrs (>20 kb)	30
Shotgun Metagenomics (10 kb)	2	30
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	10
Iso-Seq Method (standard samples)	2	24
Iso-Seq Method (focus on long transcripts)	2	24

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020

Part Number 101-769-100 Version 05 (November 2020)

For amplicon samples **≥3 kb**, PacBio recommends **Sequel II Binding Kit 2.0**.

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.

SAMPLE SETUP RECOMMENDATIONS FOR AMPLICON LIBRARIES – SEQUEL II AND IIe SYSTEMS (CHEMISTRY 2.0) (CONT.)

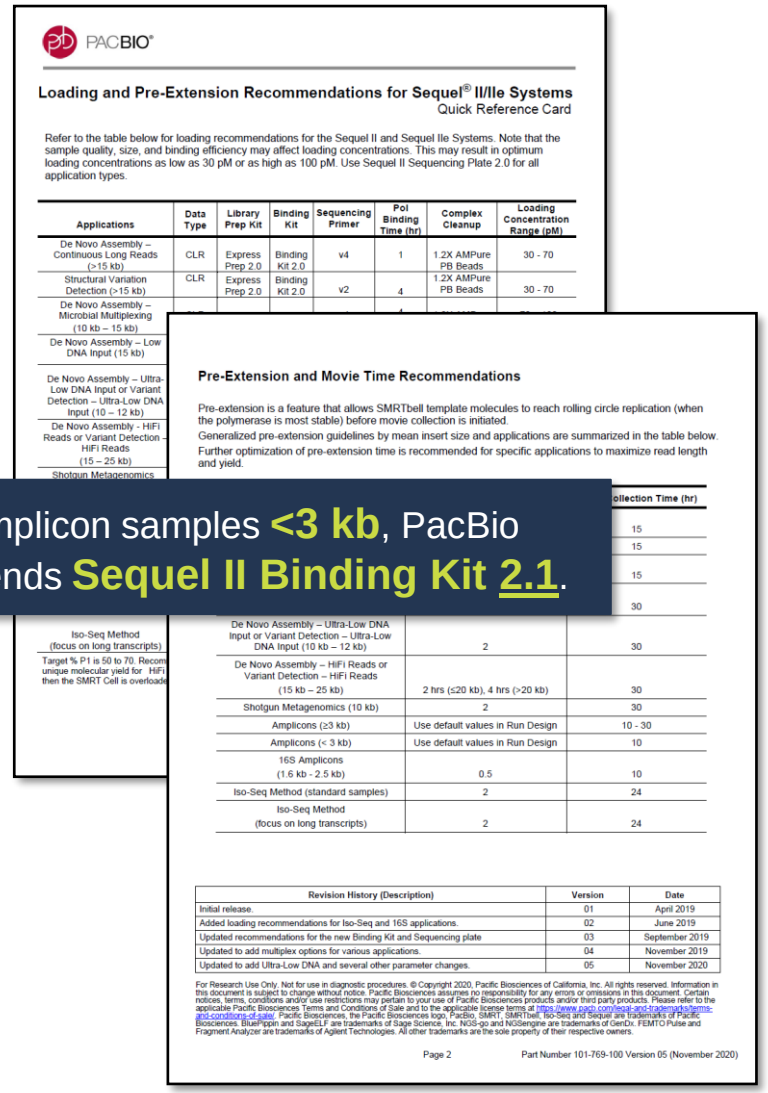
Sequel II and IIe System Sample Setup Procedure for Amplicons **<3 kb**

- Follow **SMRT Link Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems for preparing <3 kb amplicon samples for sequencing
  - For **SMRT Link v10.0** (or higher): Select **'<3 kb Amplicon Sequencing'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Amplicons (≥3 kb)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Amplicons (< 3 kb)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 150

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10

- Anticipated recovery yield after complex cleanup with AMPure PB beads*:
  - ~3 kb Amplicon Libraries: ~40%
  - ~1 – 2 kb Amplicon Libraries: ~30%


PACBIO

Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 30 pM or as high as 100 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 - 70
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)							
De Novo Assembly – Low DNA Input (15 kb)							
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 – 12 kb)							
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 – 25 kb)							
Shotgun Metagenomics (10 kb)							
Amplicons (≥3 kb)							
Amplicons (< 3 kb)							
16S Amplicons (1.6 kb - 2.5 kb)							
Iso-Seq Method (standard samples)							
Iso-Seq Method (focus on long transcripts)							

Target % P1 is 50 to 70. Recom... unique molecular yield for HiFi ... then the SMRT Cell is overloade...

Pre-Extension and Movie Time Recommendations

Pre-extension is a feature that allows SMRTbell template molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below.
Further optimization of pre-extension time is recommended for specific applications to maximize read length and yield.

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	30
De Novo Assembly – HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	2 hrs (≤20 kb), 4 hrs (>20 kb)	30
Shotgun Metagenomics (10 kb)	2	30
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	10
Iso-Seq Method (standard samples)	2	24
Iso-Seq Method (focus on long transcripts)	2	24

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020

Part Number 101-769-100 Version 05 (November 2020)

For amplicon samples **<3 kb**, PacBio recommends **Sequel II Binding Kit 2.1**.

* ProNex beads can also be used for complex cleanup and may lead to improved library recovery efficiencies for short (<3 kb) amplicon samples. To help improve accuracy of DNA quantitation of short amplicon samples during complex cleanup, use >1 µL of sample volume for Qubit assays.

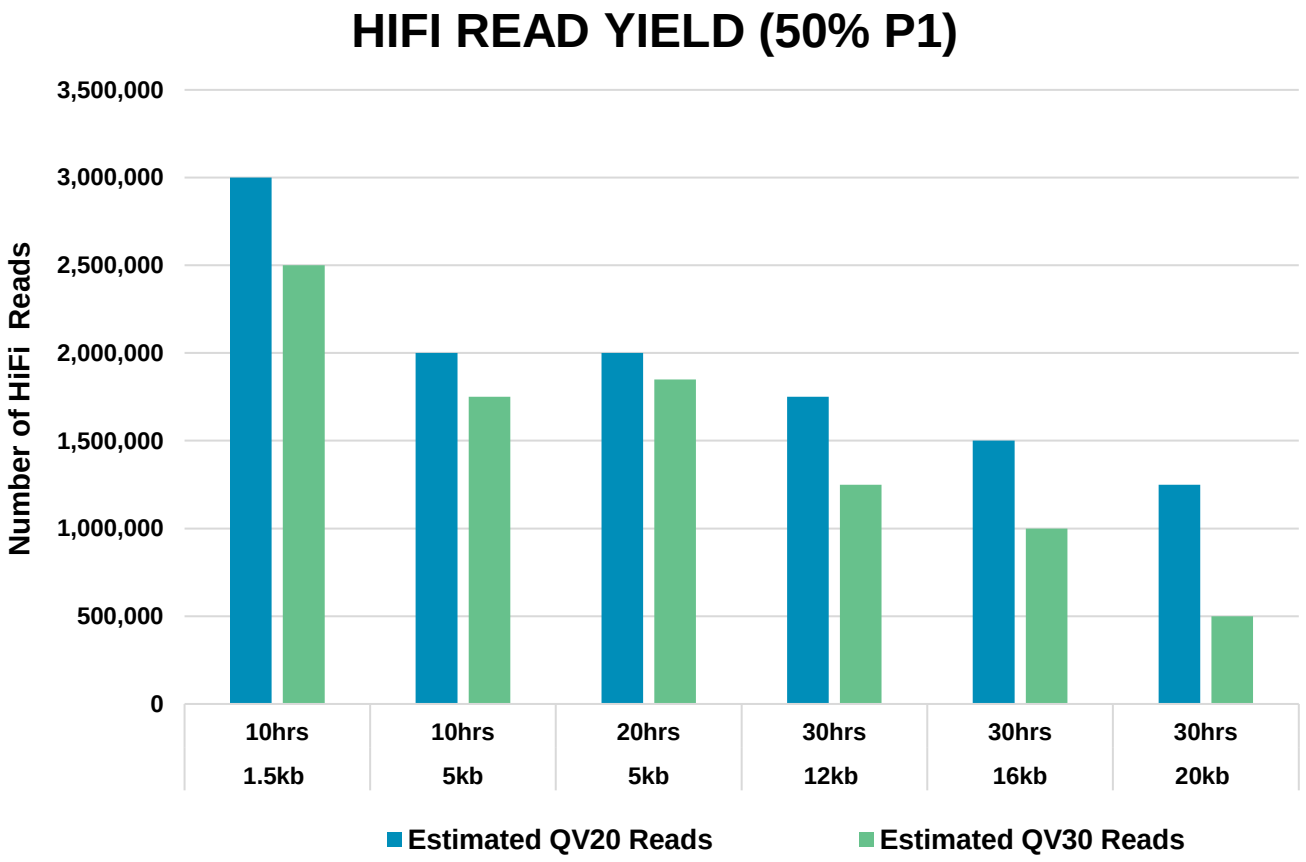
Multiplexed Amplicon Data Analysis Recommendations

DATA ANALYSIS SOLUTIONS FOR MULTIPLEXED SEQUENCING OF SMRTBELL EXPRESS TPK 2.0 AMPLICON LIBRARIES

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

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (**HiFi reads**) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- **≥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
- PacBio highly recommends upgrading to **SMRT Link v9.0 or higher** to perform de-multiplexing of your amplicon sequencing data
  - SMRT Link supports demultiplexing up to 10,000 barcoded samples per data set
  - Refer to **Barcoding Overview** documents available on PacBio's Multiplexing Resources website (https://www.pacb.com/products-and-services/analytical-software/multiplexing/) for detailed information on QC metrics for evaluation of barcoding performance using SMRT Link
    - SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)
- Can use SMRT Link to detect, quantitate, and phase single nucleotide polymorphisms within coding regions using the Minor Variants Analysis (MVA) application
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- HiFi reads are compatible with standard analysis tools for variant calling such as Google DeepVariant

HIFI (Q20, Q30 CCS) READ YIELD PERFORMANCE FOR DIFFERENT LIBRARY INSERT SIZES (SEQUEL II CHEMISTRY 2.0)



INSERT SIZE	MOVIE LENGTH	ESTIMATED ≥Q20 HIFI READS	ESTIMATED ≥Q30 HIFI READS
1.5 kb	10 hours	3,000,000	2,500,000
5 kb	10 hours	2,000,000	1,750,000
5 kb	20 hours	2,000,000	1,850,000
12 kb	30 hours	1,750,000	1,250,000
16 kb	30 hours	1,500,000	1,000,000
20 kb	30 hours	1,250,000	500,000

Data shown above are from DNA libraries constructed using the SMRTbell Express Template Prep Kit 2.0 and sequenced on a Sequel II System (Chemistry 2.0) using different movie collection times. Read lengths, reads/data per SMRT Cell 8M and other sequencing performance results vary based on sample quality/type and insert size.

Multiplexed Amplicon Library Example Sequencing Performance Data

EXAMPLE 1: BARCODED OVERHANG ADAPTER (BOA) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE

96-plex Human Amplicon Library Prepared Using PacBio-Barcoded Overhang Adapters

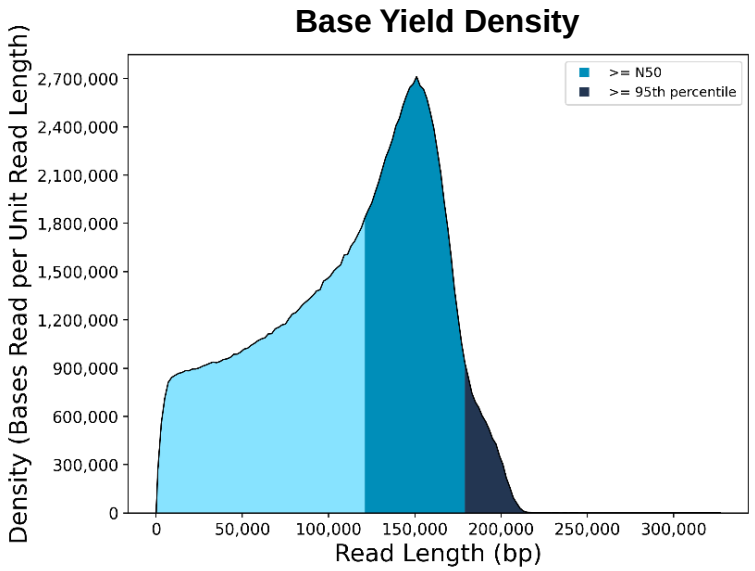
A. Example Primary Sequencing Performance Metrics (Sequel II System)

PRIMARY RUN STATISTICS* FOR A 96-PLEX HUMAN AMPLICON LIBRARY.

SAMPLE	TOTAL BASES (Gb)	UNIQUE MOLECULAR YIELD (Gb)	MEAN POLYMERASE RL (bp)	POLYMERASE RL N50 (bp)	MEAN LONGEST SUBREAD (bp)	LONGEST SUBREAD N50 (bp)	P0 (%)	P1 (%)	P2 (%)
96-Plex Human Amplicon Library (~3.1 kb – 5.7 kb)	276.8	35.4	52853	121339	7828	15667	32.0	65.5	2.6

Sequel II System Chemistry 2.0; 60 pM on-plate loading concentration; 20-hour movie collection time; 1.4-hour pre-extension time

- 16 human gDNA samples were taken into PCR amplification and all 16 were symmetrically-barcoded with 6 unique barcodes per sample to generate a 96-plex pooled SMRTbell library
  - Measured amplicon sizes for each barcoded sample ranged from ~3.1 kb to 5.7 kb
- 96-plex pooled amplicon library was sequenced on a single Sequel II SMRT Cell 8M using a 20-h movie collection time



** Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.*

EXAMPLE 1: BARCODED OVERHANG ADAPTER (BOA) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE (CONT.)

96-plex Human Amplicon Library Prepared Using PacBio-Barcoded Overhang Adapters

B. Example CCS and Barcode Demultiplexing Analysis Performance Metrics

CCS ANALYSIS SUMMARY METRICS

ANALYSIS METRIC	VALUE
HiFi (≥Q20 CCS) Reads	2,370,623
HiFi Base Yield (bp)	8,725,080,599
HiFi Read Length (mean, bp)	3,680
HiFi Read Quality (median)	Q40

- **~2.4 million HiFi reads (45%)** were obtained from ~5.2 million P1 polymerase reads generated on a single Sequel II SMRT Cell 8M
- **~2.3 million barcoded reads (97%)** were recovered after performing demultiplexing analysis of ~2.4 million input HiFi reads using SMRT Link

DEMULTIPLEXING ANALYSIS SUMMARY METRICS

ANALYSIS METRIC	VALUE
HiFi (≥Q20 CCS) Reads Input for Demultiplexing Analysis	2,370,623
Unique Barcodes Identified	96
Barcoded HiFi Reads	2,288,576
Unbarcoded Reads	82,047
Mean HiFi Reads per Barcode	23,839
Max. HiFi Reads per Barcode	38,905
Min. HiFi Reads per Barcode	12,469
Mean HiFi Read Length (bp) of Barcoded Reads	3,671

Sequel II System Chemistry 2.0; 60 pM on-plate loading concentration; 20-hour movie collection time; 1.4-hour pre-extension time; SMRT Link v9.0.

EXAMPLE 2: BARCODED UNIVERSAL PRIMER (BUP) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE

92-plex Bacterial Amplicon Library Prepared Using PacBio-Barcoded M13 Universal Primers

A. Example Primary Sequencing Performance Metrics (Sequel II System)

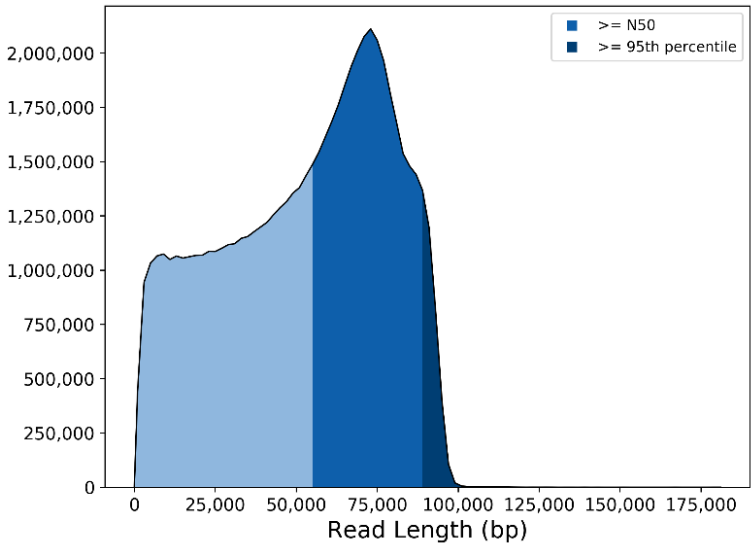
PRIMARY RUN STATISTICS* FOR A 92-PLEX BACTERIAL AMPLICON LIBRARY

SAMPLE	TOTAL BASES (Gb)	UNIQUE MOLECULAR YIELD (Gb)	MEAN POLYMERASE RL (bp)	POLYMERASE RL N50 (bp)	MEAN LONGEST SUBREAD (bp)	LONGEST SUBREAD N50 (bp)	P0 (%)	P1 (%)	P2 (%)
92-Plex Bacterial Amplicon Library (~2.6 kb)	128.7	14.9	23662	56425	3086	2733	27.0	67.9	5.2

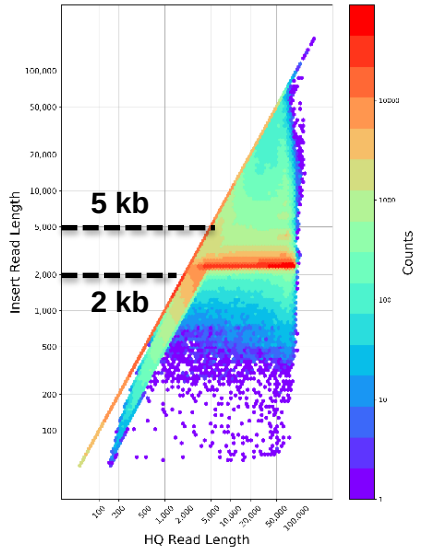
Sequel II System Chemistry 2.0; 50 pM on-plate loading concentration; 10-hour movie collection time; 0.6-hour pre-extension time

- Amplicons were generated using *E. coli* gDNA
  - Measured amplicon size is ~2.6 kb
- Replicate amplicon samples were asymmetrically-barcoded with 96 unique barcoded M13 universal primer pairs to generate a 92-plex pooled SMRTbell library
- 92-plex pooled bacterial amplicon library was sequenced on a single Sequel II SMRT Cell 8M using a 10-h movie collection time

Base Yield Density



Insert Read Length Density



** Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.*

EXAMPLE 2: BARCODED UNIVERSAL PRIMER (BUP) MULTIPLEXED AMPLICON SEQUENCING PERFORMANCE (CONT.)

92-plex Bacterial Amplicon Library Prepared Using PacBio-Barcoded M13 Universal Primers

B. Example CCS and Barcode Demultiplexing Analysis Performance Metrics

CCS ANALYSIS SUMMARY METRICS

ANALYSIS METRIC	VALUE
HiFi (≥Q20 CCS) Reads	2,435,305
HiFi Base Yield (bp)	5,513,188,488
HiFi Read Length (mean, bp)	2,263
HiFi Read Quality (median)	Q40

- ~**2.4 million HiFi reads (45%)** were obtained from ~5.4 million P1 polymerase reads generated on a single Sequel II SMRT Cell 8M
- **~2.3 million barcoded reads (95%)** were recovered after performing demultiplexing analysis of ~2.4 million input HiFi reads using SMRT Link

DEMULTIPLEXING ANALYSIS SUMMARY METRICS

ANALYSIS METRIC	VALUE
HiFi (≥Q20 CCS) Reads Input for Demultiplexing Analysis	**2,435,305**
Unique Barcodes Identified	**92**
Barcoded HiFi Reads	**2,311,437**
Unbarcoded Reads	**123,868**
Mean HiFi Reads per Barcode	**25,124**
Max. HiFi Reads per Barcode	**50,999**
Min. HiFi Reads per Barcode	**22,429**
Mean HiFi Read Length (bp) of Barcoded Reads	**2,243**

Sequel II System Chemistry 2.0; 50 pM on-plate loading concentration; 10-hour movie collection time; 0.6-hour pre-extension time; SMRT Link v9.0.

Technical Documentation & Applications Support Resources

BEST PRACTICES: TARGETED SEQUENCING FOR AMPLICONS

LIBRARY PREP

Template Preparation with SMRTbell Express Template Prep Kit 2.0

- Start with high-quality, double-stranded DNA for PCR amplification
- Create SMRTbell templates between ~250 bp to 20 kb from ≥10 ng input mass per amplicon
- Optimize data throughput with flexible barcoding options:
  - Amplify PCR products using target-specific primers with incorporated barcodes; OR
  - Attach Barcoded Overhang Adapters during ligation without modifying existing primers; OR
  - Add Barcoded Universal Primers into amplicons via a simple 2-step PCR process

SMRT SEQUENCING

Sequence on the Sequel, Sequel II or Sequel IIe Systems

- Maximize output and turn-around-time with adjustable run parameters
  - For inserts <5 kb, recommend 10-hour movie collection time; for inserts ≥5 kb, recommend 20-hour or longer movie collection time
- Generate up to ~3 million HiFi reads per Sequel II System SMRT Cell 8M*
- Sequence to desired coverage based on project needs
  - ≥50-fold coverage of HiFi reads per target locus is recommended for variant detection
  - 6,000-fold HiFi read coverage per target locus is recommended for minor variant detection (1% sensitivity)

DATA ANALYSIS

Data Analysis Solutions with the PacBio Analytical Portfolio

- Fully characterize genetic complexity – structural variation, rare SNPs, indels, CNV, microsatellites, haplotypes, and phasing
- Utilize a variety of analysis tools within SMRT Link
  - Generate highly accurate HiFi single-molecule reads using Circular Consensus Sequencing (CCS)
  - Detect, quantitate, and phase single nucleotide polymorphisms within coding regions using Minor Variants Analysis
- Easily de-multiplex barcodes within SMRT Link
- Output data in standard file formats for integration with downstream analysis tools
- HiFi long reads are compatible with standard analysis tools for variant calling such as Google DeepVariant

** Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED AMPLICON LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS

Sample Preparation Literature

- Application Brief: Targeted Sequencing for Amplicons – Best Practices (PN BP105-071919)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplex SMRT Sequencing (PN 101-791-700)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded Universal Primers for Multiplex SMRT Sequencing (PN 101-791-800)
- Procedure & Checklist – Preparing SMRTbell Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT Sequencing (PN 101-921-300)
- Reference Guide – Human Leukocyte Antigen (HLA) Sequencing (PN 102-066-200)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel System (PN 101-461-600)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II System (PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Consumable Bundles Purchasing Guide (PN PG100-051320)
- Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (PN TN101-061920)
- Technical Overview: Multiplexed Amplicon Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-814-300)

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED AMPLICON LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Data Analysis Resources

- SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)
  - Contains detailed information on barcoding experimental design options and describes QC metrics for evaluation of barcoding performance using SMRT Link
- PacBio Multiplexing Resources Website: https://www.pacb.com/smrt-science/smrt-sequencing/multiplexing/
  - Barcoding Overview documents for different SMRT Link software versions
  - PacBio barcode sequence files (compressed FASTA) for use with Sequel, Sequel II and Sequel IIe Systems
  - Barcoded oligo ordering sheets

Example PacBio Data Sets

TARGETED SEQUENCING APPLICATION	DATASET	DATA TYPE	PACBIO SYSTEM
HLA Sequencing	Analysis of HLA Amplicons (*HLA-A, -B, -C, -DRB1, -DQB1* and –*DPB1*) Generated Using GenDX NGSgo-MX6-1 Kit	HiFi Reads	Sequel II System
Viral Sequencing	SARS-CoV-2 – ATCC Control with Eden Primers	HiFi Reads	Sequel II System

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR MULTIPLEXED AMPLICON LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Posters

- ASHG 2019 Poster: The value of long read amplicon sequencing for clinical applications.
- ASHG 2019 Poster: TLA & long-read sequencing: Efficient targeted sequencing and phasing of the CFTR gene
- ASHG 2018 Poster: A simple segue from Sanger to high-throughput SMRT Sequencing with a M13 barcoding system
- ESHG 2018 Poster: High-throughput SMRT Sequencing of clinically relevant targets

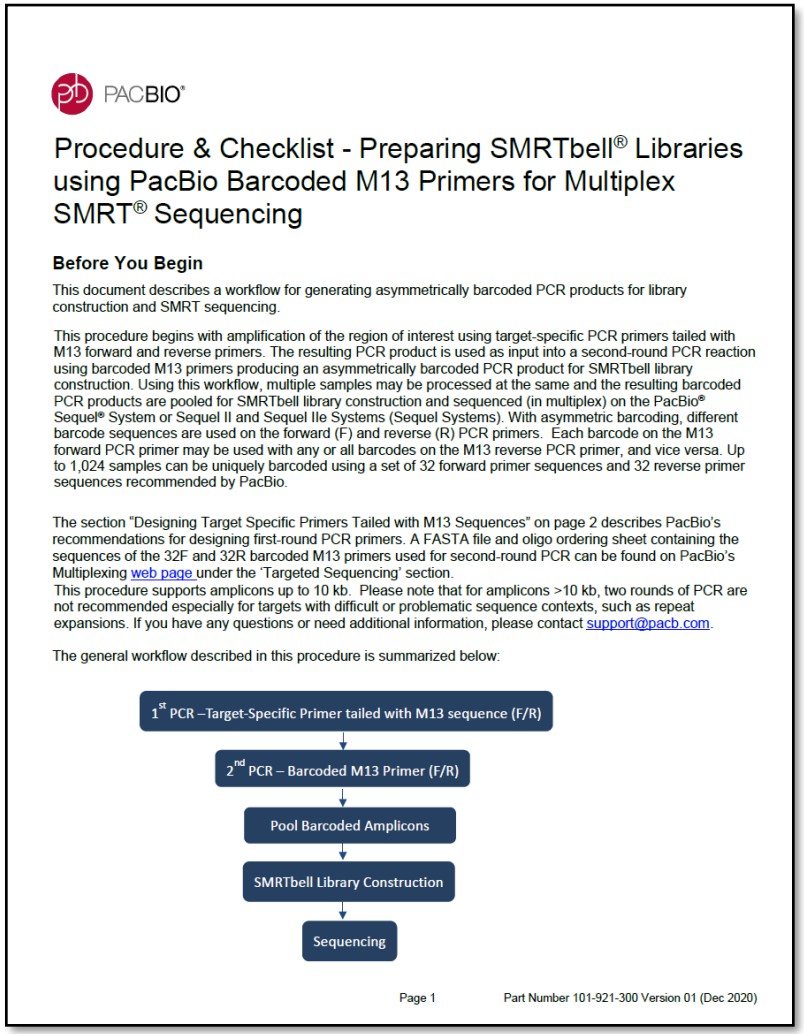
Publications

- Botton, Mariana R. et al. (2020) Phased Haplotype Resolution of the SLC6A4 Promoter Using Long-Read Single Molecule Real-Time (SMRT) Sequencing. Genes. 11(11):1333.
- Vasan, Neil et al. (2019) Double PIK3CA mutations in cis increase oncogenicity and sensitivity to PI3Ka inhibitors. Science. 366(6466):714–723.
- Mayor, N. P., et al. (2019) Recipients receiving better HLA-matched hematopoietic cell transplantation grafts, uncovered by a novel HLA typing method, have superior survival: A retrospective study. Biology of Blood and Marrow Transplantation. 25(3):443–450.
- Van de Weyer, A.-L., et al. (2019). A Species-Wide Inventory of NLR Genes and Alleles in Arabidopsis thaliana. Cell. 178(5):1260–1272.e14.
- Wenzel, A. et al., (2018) Single molecule real time sequencing in ADTKD-MUC1 allows complete assembly of the VNTR and exact positioning of causative mutations. Scientific Reports. 8(1):4170.

Appendix 1: Multiplexed Amplicon Library Preparation Using PacBio Barcoded M13 Primers

PROCEDURE & CHECKLIST – PREPARING SMRTBELL LIBRARIES USING PACBIO BARCODED M13 PRIMERS FOR MULTIPLEX SMRT SEQUENCING

- This protocol (PN 101-921-300) describes a 2-step PCR method for generating up to 1,024 asymmetrically barcoded amplicon products using **barcoded M13 primers** and performing SMRTbell library construction for multiplexed amplicon sequencing on Sequel, Sequel II and Sequel IIe Systems (Sequel Systems)
- Protocol document contains:
  1. Best practices recommendations for generating high-quality PCR products for PacBio sequencing
  2. Instructions for generating **asymmetrically barcoded amplicons** using a 2-Step PCR method with customer-supplied (forward & reverse) barcoded M13 primers and performing equimolar pooling of barcoded amplicon products
  3. Instructions for constructing SMRTbell libraries with pooled barcoded PCR amplicons using PacBio's SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing multiplexed amplicon SMRTbell libraries for sequencing on the Sequel Systems
- This procedure supports amplicon sizes up to 10 kb.
  - Note that for amplicons >10 kb, two rounds of PCR are **not** recommended especially for targets with difficult or problematic sequence contexts, such as repeat expansions.



PACBIO®

Procedure & Checklist - Preparing SMRTbell® Libraries using PacBio Barcoded M13 Primers for Multiplex SMRT® Sequencing

Before You Begin

This document describes a workflow for generating asymmetrically barcoded PCR products for library construction and SMRT sequencing.

This procedure begins with amplification of the region of interest using target-specific PCR primers tailed with M13 forward and reverse primers. The resulting PCR product is used as input into a second-round PCR reaction using barcoded M13 primers producing an asymmetrically barcoded PCR product for SMRTbell library construction. Using this workflow, multiple samples may be processed at the same and the resulting barcoded PCR products are pooled for SMRTbell library construction and sequenced (in multiplex) on the PacBio® Sequel® System or Sequel II and Sequel IIe Systems (Sequel Systems). With asymmetric barcoding, different barcode sequences are used on the forward (F) and reverse (R) PCR primers. Each barcode on the M13 forward PCR primer may be used with any or all barcodes on the M13 reverse PCR primer, and vice versa. Up to 1,024 samples can be uniquely barcoded using a set of 32 forward primer sequences and 32 reverse primer sequences recommended by PacBio.

The section "Designing Target Specific Primers Tailed with M13 Sequences" on page 2 describes PacBio's recommendations for designing first-round PCR primers. A FASTA file and oligo ordering sheet containing the sequences of the 32F and 32R barcoded M13 primers used for second-round PCR can be found on PacBio's Multiplexing web page under the 'Targeted Sequencing' section.
This procedure supports amplicons up to 10 kb. Please note that for amplicons >10 kb, two rounds of PCR are not recommended especially for targets with difficult or problematic sequence contexts, such as repeat expansions. If you have any questions or need additional information, please contact support@pacb.com.

The general workflow described in this procedure is summarized below:

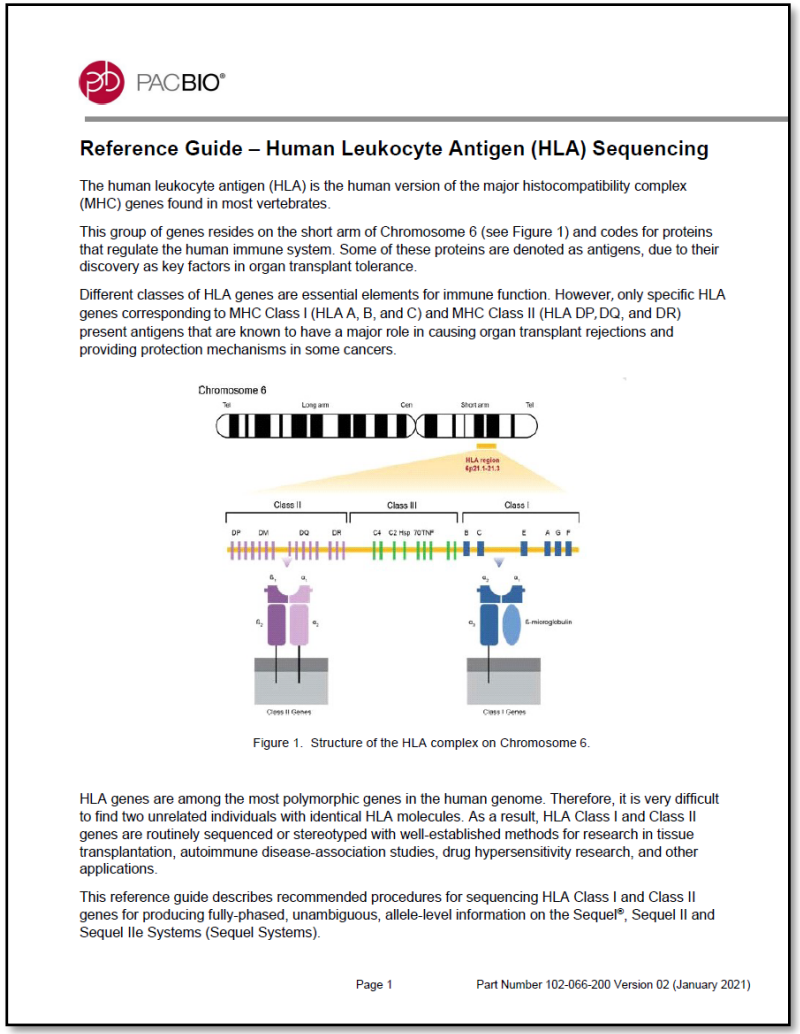
Page 1 Part Number 101-921-300 Version 01 (Dec 2020)

https://www.pacb.com/support/documentation/

Appendix 2: Sample Preparation Recommendations for HLA Amplicon SMRT Sequencing

REFERENCE GUIDE – HUMAN LEUKOCYTE ANTIGEN (HLA) SEQUENCING

- This reference document (PN 102-066-200) describes recommended procedures for sequencing HLA Class I and Class II genes on the Sequel, Sequel II and Sequel IIe Systems for producing fully-phased, unambiguous, allele-level information.
- Reference guide contains:
  1. Best practices recommendations for input genomic DNA QC and generating high-quality PCR products for PacBio sequencing using either commercial PCR primers (GenDx HLA Typing Kit) or customer-designed PCR primers.
  2. Recommendations for planning multiplexing experiments using Barcoded Overhang Adapters[1] (PN 101-791-700), Barcoded Universal Primers[2] (PN 101-791-800), or Barcoded M13 Primers[3] (PN 101-921-300)
  3. Workflow summary overview for constructing barcoded HLA SMRTbell libraries with input PCR amplicons using PacBio's SMRTbell Express Template Prep Kit 2.0
  4. Sample setup guidance for preparing multiplexed HLA amplicon SMRTbell libraries for sequencing on the Sequel, Sequel II and Sequel IIe Systems
- If you are using a **commercial GenDx HLA Typing Kit** to prepare HLA amplicons for analysis, refer to **Part A** of this Reference Guide for recommended procedures for constructing a multiplexed SMRTbell library for SMRT Sequencing.
- If using your own **custom-designed PCR primers** to amplify HLA genes for analysis, proceed directly to **Part B** of this Reference Guide for sample preparation guidance.



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Reference Guide – Human Leukocyte Antigen (HLA) Sequencing

The human leukocyte antigen (HLA) is the human version of the major histocompatibility complex (MHC) genes found in most vertebrates.

This group of genes resides on the short arm of Chromosome 6 (see Figure 1) and codes for proteins that regulate the human immune system. Some of these proteins are denoted as antigens, due to their discovery as key factors in organ transplant tolerance.

Different classes of HLA genes are essential elements for immune function. However, only specific HLA genes corresponding to MHC Class I (HLA A, B, and C) and MHC Class II (HLA DP, DQ, and DR) present antigens that are known to have a major role in causing organ transplant rejections and providing protection mechanisms in some cancers.

Figure 1. Structure of the HLA complex on Chromosome 6.

HLA genes are among the most polymorphic genes in the human genome. Therefore, it is very difficult to find two unrelated individuals with identical HLA molecules. As a result, HLA Class I and Class II genes are routinely sequenced or stereotyped with well-established methods for research in tissue transplantation, autoimmune disease-association studies, drug hypersensitivity research, and other applications.

This reference guide describes recommended procedures for sequencing HLA Class I and Class II genes for producing fully-phased, unambiguous, allele-level information on the Sequel®, Sequel II and Sequel IIe Systems (Sequel Systems).

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[1] Barcoded Overhang Adapter Kits 8A & 8B support multiplexing of up to 16 samples. If multiplexing projects require more than 16 samples (up to 96 samples), we offer a list of barcoded overhang adapter sequences that you can order from an oligo synthesis provider.

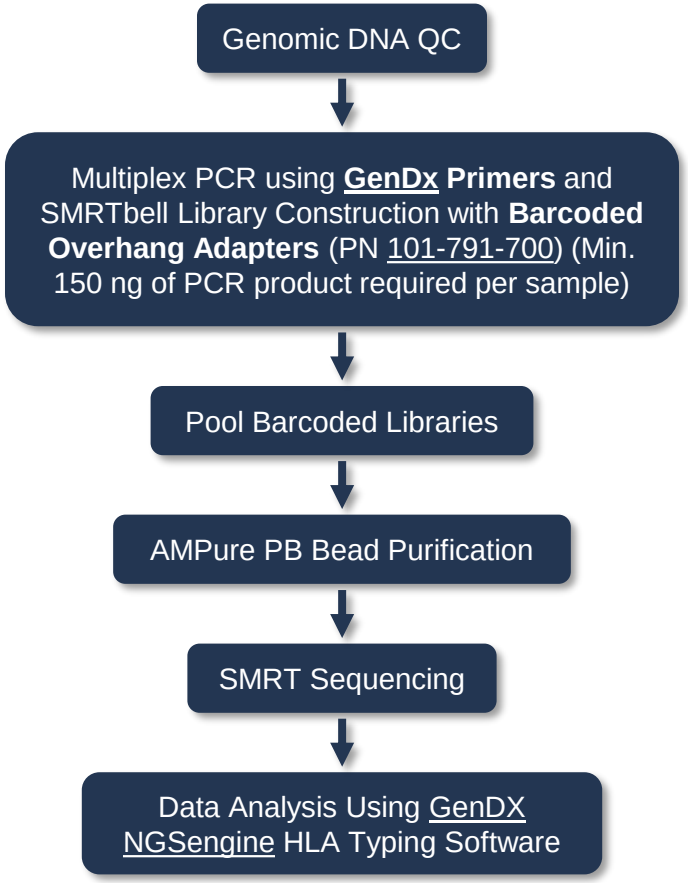
[2] PacBio Barcoded Universal Primers kit (PN 101-629-100) supports generating up to 96 symmetrically barcoded amplicon product.

[3] Barcoded M13 Primers protocol supports generating up to 1,024 asymmetrically barcoded amplicon products using barcoded M13 primers.

https://www.pacb.com/support/documentation/

SMRTBELL LIBRARY PREPARATION WORKFLOW OVERVIEW FOR HLA AMPLICON SAMPLES GENERATED WITH GenDX HLA TYPING KITS

GenDx (gendx.com) offers validated HLA primers with ready-to-use PCR master mixes, protocols and tools for SMRT Sequencing on Sequel, Sequel II and Sequel IIe Systems



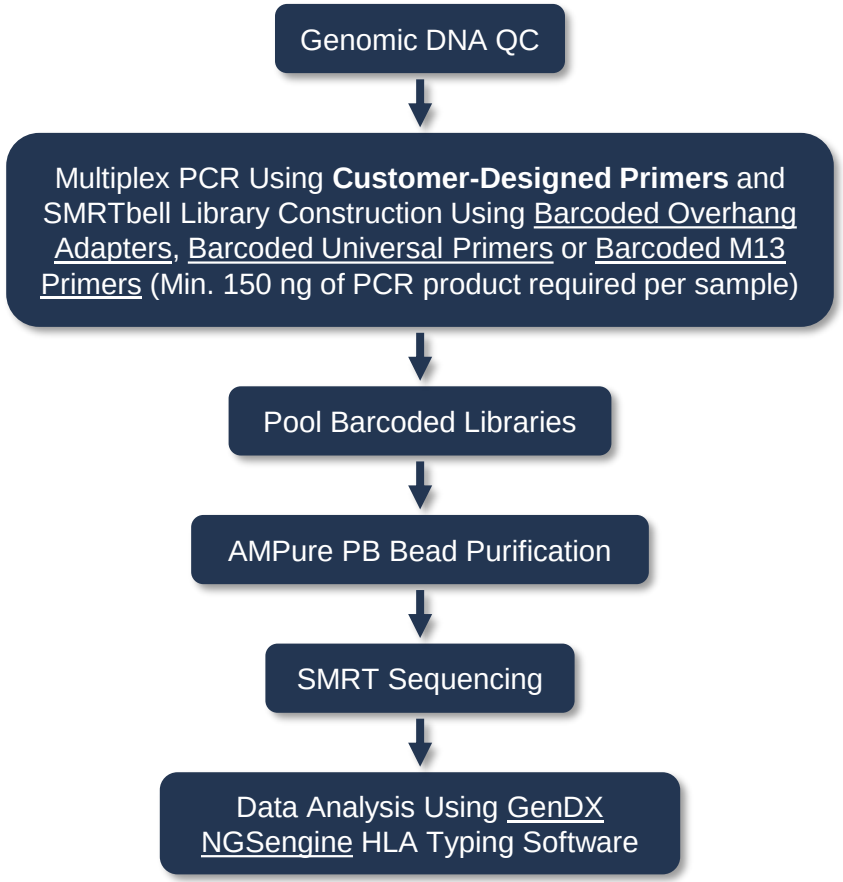
Workflow for amplification and sequencing of HLA amplicons generated with GenDx PCR primers. For procedure details, refer to ***Reference Guide – HLA Sequencing*** (PN 102-066-200).

- Depending on your project requirements, 3 kits are available from GenDX for typing up to 11 HLA loci
- PacBio has validated the GenDx NGSgo-MX6-1 Kit
  - Features six HLA loci in one tube for the amplification of HLA-A, -B, -C, -DRB1, -DQB1 and –DPB1 (3 – 6 kb target amplicon sizes)
  - Kit consists of 3 reagent tubes supporting up to 96 PCR reactions total:
    - NGSgo-MX6-1 primer mix
    - GenDx-LongMix PCR master mix (4x)
    - Nuclease-free water

- To reduce cost per sample, multiple samples may be barcoded using Barcoded Overhang Adapters and pooled for sequencing on a single SMRT Cell
  - Up to 48 barcoded samples may be pooled for sequencing on the Sequel System and up to 96 barcoded samples may be pooled for sequencing on the Sequel II and IIe Systems
  - Refer to ***Procedure & Checklist - Preparing SMRTbell Libraries using PacBio Barcoded Overhang Adapters for Multiplexing Amplicons*** (PN 101-791-700) for barcoding protocol details
- HLA typing analysis of HiFi reads can be performed with GenDx NGSengine software

SMRTBELL LIBRARY PREPARATION WORKFLOW OVERVIEW FOR HLA AMPLICON SAMPLES GENERATED WITH CUSTOMER-DESIGNED ASSAYS

HLA amplicon samples generated with **customer-designed assays** can be constructed to SMRTbell libraries and sequenced on Sequel, Sequel II and Sequel IIe Systems



Workflow for amplification and sequencing of HLA amplicons generated with customer-designed PCR primers. For procedure details, refer to ***Reference Guide – HLA Sequencing*** (PN 102-066-200).

- **NOTE:** Users may design their own custom primers to amplify HLA gene targets for SMRT Sequencing – but be aware that painstaking and time-consuming validation experiments may be required to develop an optimized sample preparation workflow.
- **We therefore highly recommend using validated and ready-to-use primers from GenDx to amplify HLA gene targets for PacBio sequencing** (See **Part A** of ***Reference Guide – HLA Sequencing*** (PN 102-066-200).

- To reduce cost per sample, multiple samples may be barcoded and pooled for sequencing on a single SMRT Cell
  - Up to 48 barcoded samples may be pooled for sequencing on the Sequel System and up to 96 barcoded samples may be pooled for sequencing on the Sequel II and IIe Systems
  - Three barcoding strategies are available; see Procedure & Checklist protocols for **Barcoded Overhang Adapters** (PN 101-791-700), **Barcoded Universal Primers** (PN 101-791-800) or **Barcoded M13 Primers** (PN 101-921-300)
- HLA typing analysis of HiFi reads can be performed with GenDx NGSengine software

SAMPLE SETUP RECOMMENDATIONS FOR SEQUENCING HLA AMPLICON LIBRARIES

SMRT Link Sample Setup provides guidance on how to anneal the sequencing primer, bind the sequencing polymerase and clean up HLA amplicon samples for loading on PacBio sequencing systems.

SMRT Link Sample Setup Conditions for Primer Annealing and Polymerase Binding

SMRT LINK VERSION	SAMPLE SETUP PARAMETER	SEQUEL SYSTEM	SEQUEL II AND IIE SYSTEMS
v10.0	Application	N/A	Select '≥3 kb Amplicons'
v9.0 / v10.0	Sequencing Primer	Sequencing Primer v4	Sequencing Primer v4
	Primer:Template Ratio	20:1	20:1
	Binding Kit	Sequel Binding Kit 3.0	Sequel II Binding Kit 2.0
	Polymerase: Template Ratio	30:1	30:1
	Loading Concentration (pM)	5	50 – 60

SMRT Link Run Design Settings and Recommended Sequencing Run Parameters

SMRT LINK VERSION	RUN DESIGN PARAMETER	SEQUEL SYSTEM	SEQUEL II AND IIE SYSTEMS
v10.0	Application	N/A	Select '≥3 kb Amplicons'
	Generate HiFi Reads	N/A	Select 'On-Instrument' or 'In SMRT Link'
v9.0	Sequencing Mode	Select 'CCS Reads'	Select 'CCS Reads'
	Generate CCS Data	Select 'Yes' to enable auto-CCS analysis	Select 'Yes' to enable auto-CCS analysis
v9.0 / v10.0	Pre-Extension Time (hours)	1.1	1.1
	Movie Collection Time (hours)	20	20

HLA DATA ANALYSIS WORKFLOW RECOMMENDATIONS

PacBio recommends using GenDx's NGSengine software for HLA typing.

1. **Generate HiFi Reads**
2. **Demultiplex Barcodes**
3. **GenDX NGSengine HLA Typing Software**

1. Utilize SMRT Link to generate highly accurate and long single-molecule reads (**HiFi reads**) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
   - → In SMRT Link, recommend setting Minimum CCS Read Length = 3000 bp
2. Utilize SMRT Link **Demultiplex Barcodes** analysis application to separate HiFi reads by sample barcode
   - → Barcode FASTA files for demultiplexing can be downloaded from PacBio's Multiplexing website
3. Utilize **GenDX NGSengine** software to perform HLA Typing analysis of de-multiplexed HiFi data
   - → Recommended Input: **≥100 HiFi reads per HLA locus**
   - → NGSengine accepts as input batches of demultiplexed FASTQ files containing HiFi sequencing data for 1 or more pooled HLA loci.
   - → HLA locus assignment and HLA typing for each demultiplexed sample dataset can be performed on a Windows-based laptop computer.

An **example demultiplexed HLA dataset analyzed using NGSengine** is available for download from PacBio (Link)

https://www.gendx.com/product_line/ngsengine/

EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS

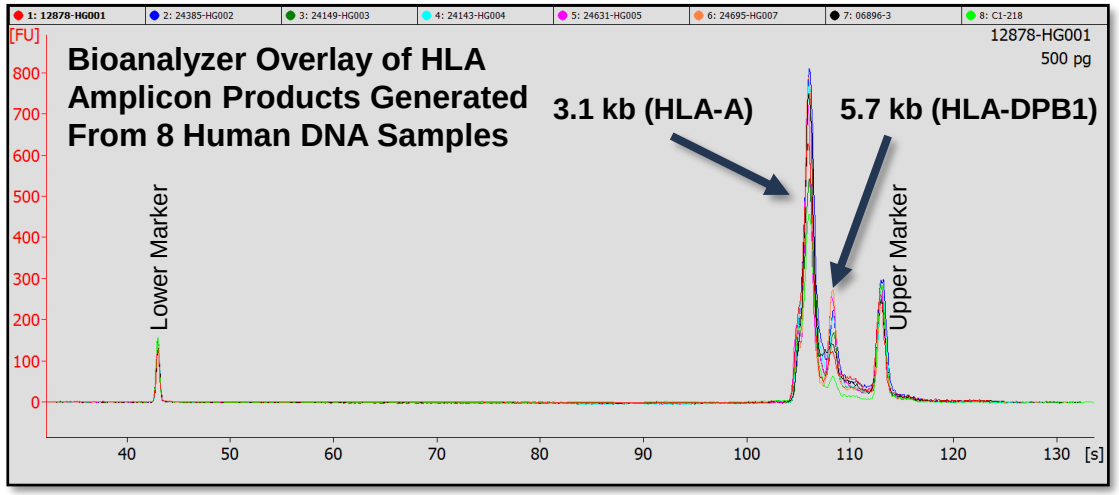
A. HLA Amplicon Generation Using GenDX NGSgo-MX6-1 Kit

PCR PRODUCT YIELDS FOR 8 HUMAN gDNA SAMPLES AMPLIFIED USING THE GenDX NGSgo-MX6-1 KIT.

Sample ID	NIST ID	# of Replicate PCR Reactions*	Purified PCR Product Conc. (ng/µl)	Purified PCR Product Volume (µl)	Total Mass of Purified PCR Products (ng)	Mass of Purified PCR Product per Reaction (ng)
NA12878	HG001	4	228	16	3,648	912
NA24385	HG002	4	250	16	4,000	1,000
NA24149	HG003	4	240	16	3,840	960
NA24143	HG004	4	221	16	3,392	848
NA24631	HG005	4	250	16	4,000	1,000
NA24695	HG007	4	252	16	4,032	1,008
NA06896	N/A	4	254	16	4,064	1,016
C1-218	N/A	4	230	16	3,680	912

* For each PCR reaction, 200 ng of input gDNA per sample was used. The fragment size of the input genomic DNA samples ranged from 22 kb to 147 kb (mode).

- Four (4) replicate PCR reactions were performed for each human gDNA sample (input gDNA size mode ~22 kb – 147 kb).
- For each sample, replicate PCR reaction products were pooled and purified with 0.6X AMPure PB beads
- Bioanalyzer sizing QC results are consistent with expected range of PCR amplicon sizes (~3.1 – 5.7 kb) for these HLA samples



EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

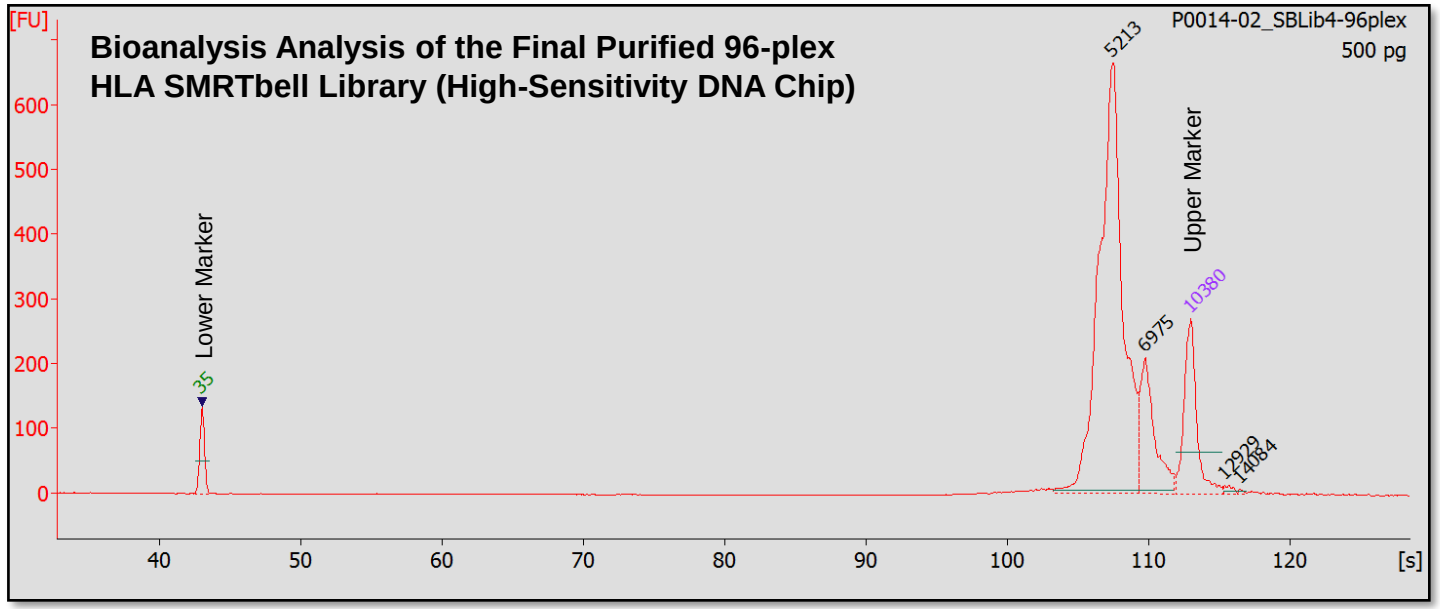
B. HLA Amplicon SMRTbell Library Construction Using SMRTbell Express Template Prep Kit 2.0

96-PLEX HLA SMRTBELL LIBRARY CONSTRUCTION YIELD

SMRTbell Library	Number of Pooled Samples	Input Mass of PCR DNA per Sample for Library Construction	Total Input Mass	Purified Library Conc. (ng/µl)*	Purified Library Volume (µl)	Purified Library Mass (ng)	Library Construction Yield (%)
96-Plex HLA Library	96	150	14,400	135	96	12,960	90

* The final 96-plex HLA SMRTbell library was purified using two rounds of 0.6X AMPure PB bead purification at the end of the procedure

- Each of eight (8) human gDNA samples that was amplified using the GenDX NGSgo-MX6-1 kit was symmetrically barcoded with 12 unique Barcoded Overhang Adapters to generate a 96-plex pooled HLA SMRTbell library for sequencing on a Sequel II System
- Mean size of the final purified 96-plex HLA SMRTbell library was ~5700 bp



EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

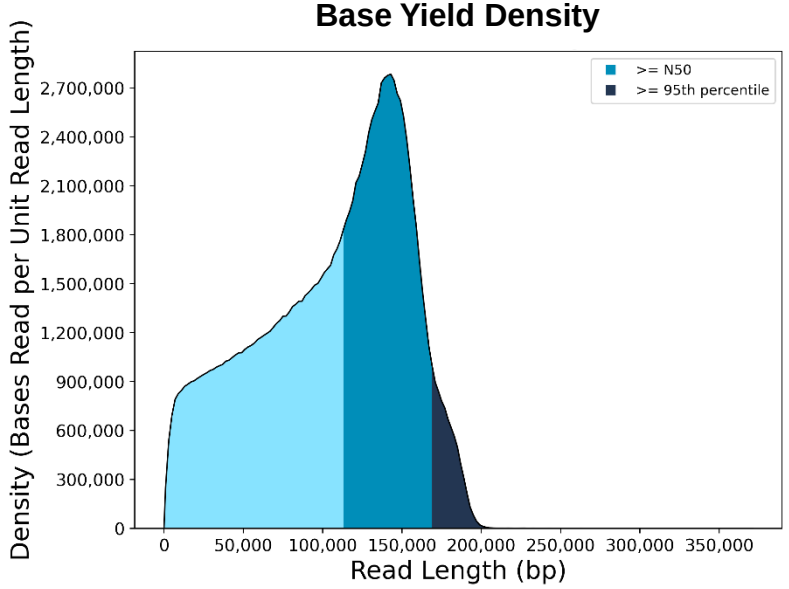
C. HLA Amplicon SMRTbell Library Primary Sequencing Performance Metrics (Sequel II System)

PRIMARY RUN STATISTICS* FOR A 96-PLEX HLA SMRTBELL LIBRARY

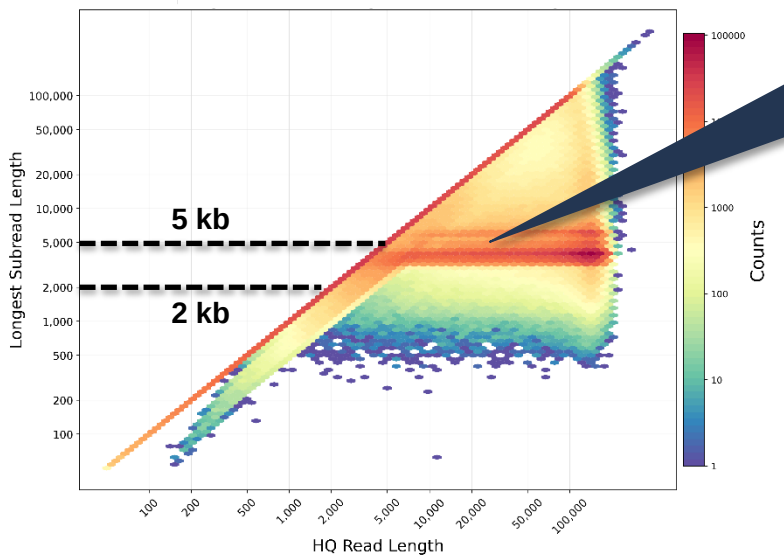
SMRTBELL LIBRARY	TOTAL BASES (Gb)	UNIQUE MOLECULAR YIELD (Gb)	MEAN POLYMERASE RL (bp)	POLYMERASE RL N50 (bp)	MEAN LONGEST SUBREAD (bp)	LONGEST SUBREAD N50 (bp)	P0 (%)	P1 (%)	P2 (%)
96-Plex HLA Library	269.72	45.57	51900	114769	9797	29150	32.3	65	2.8

Sequel II System Chemistry 2.0; 54 pM on-plate loading concentration; 20-hour movie collection time; 1.1-hour pre-extension time

Base Yield Density



Insert Read Length Density



IRLD Plot heat map is consistent with expected range of insert sizes (~3.1 – 5.7 kb) for this pooled HLA amplicon library

** Read lengths, reads/data per SMRT Cell and other sequencing performance results vary based on sample quality/type and insert size.*

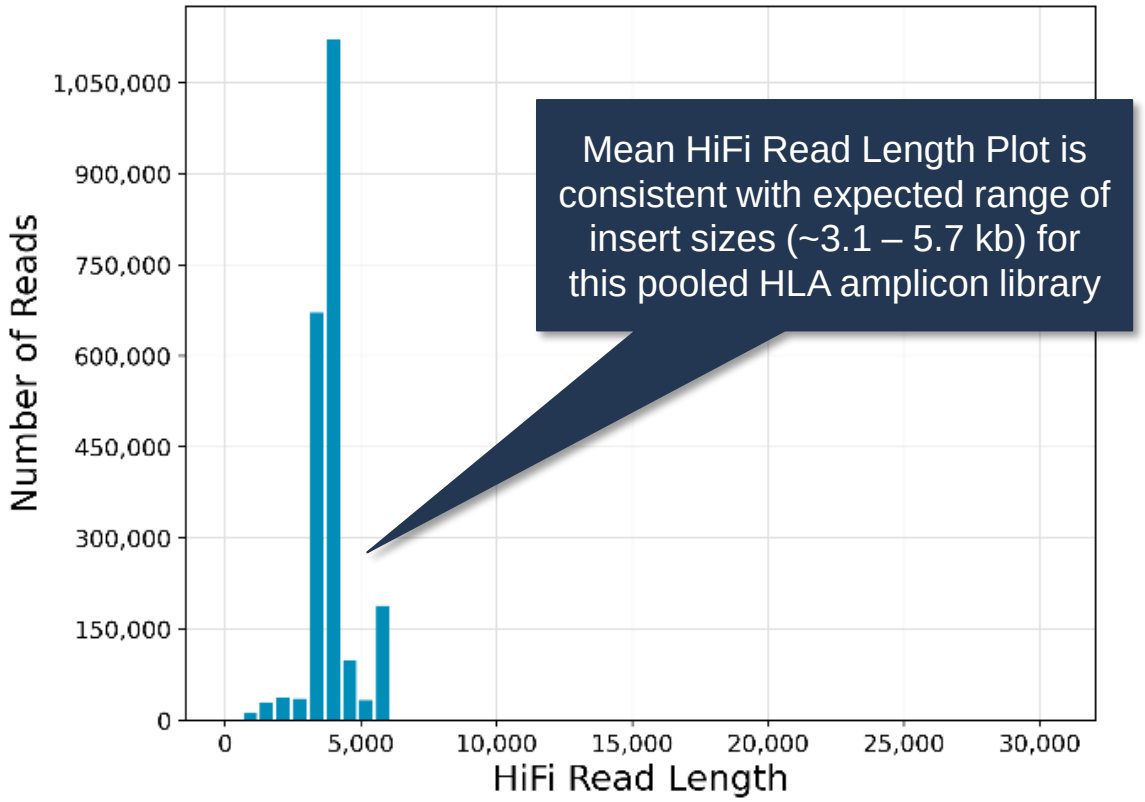
EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

D. HLA Amplicon SMRTbell Library CCS Analysis and Barcode Demultiplexing Results (Sequel II System)

96-PLEX HLA SMRTBELL LIBRARY CCS ANALYSIS AND BARCODE DEMULTIPLEXING RESULTS

ANALYSIS*	ANALYSIS METRIC	VALUE
CCS	HiFi (≥Q20 CCS) Reads	2,229,796
Demultiplex Barcodes	Unique Barcodes Detected	96
	Total Barcoded HiFi Reads	2,162,071
	Barcode Recovery Rate	97%
	Mean Barcoded HiFi Reads per Sample	22,521
	Max. Barcoded HiFi Reads per Sample	33,036
	Min. Barcoded HiFi Reads per Sample	12,045

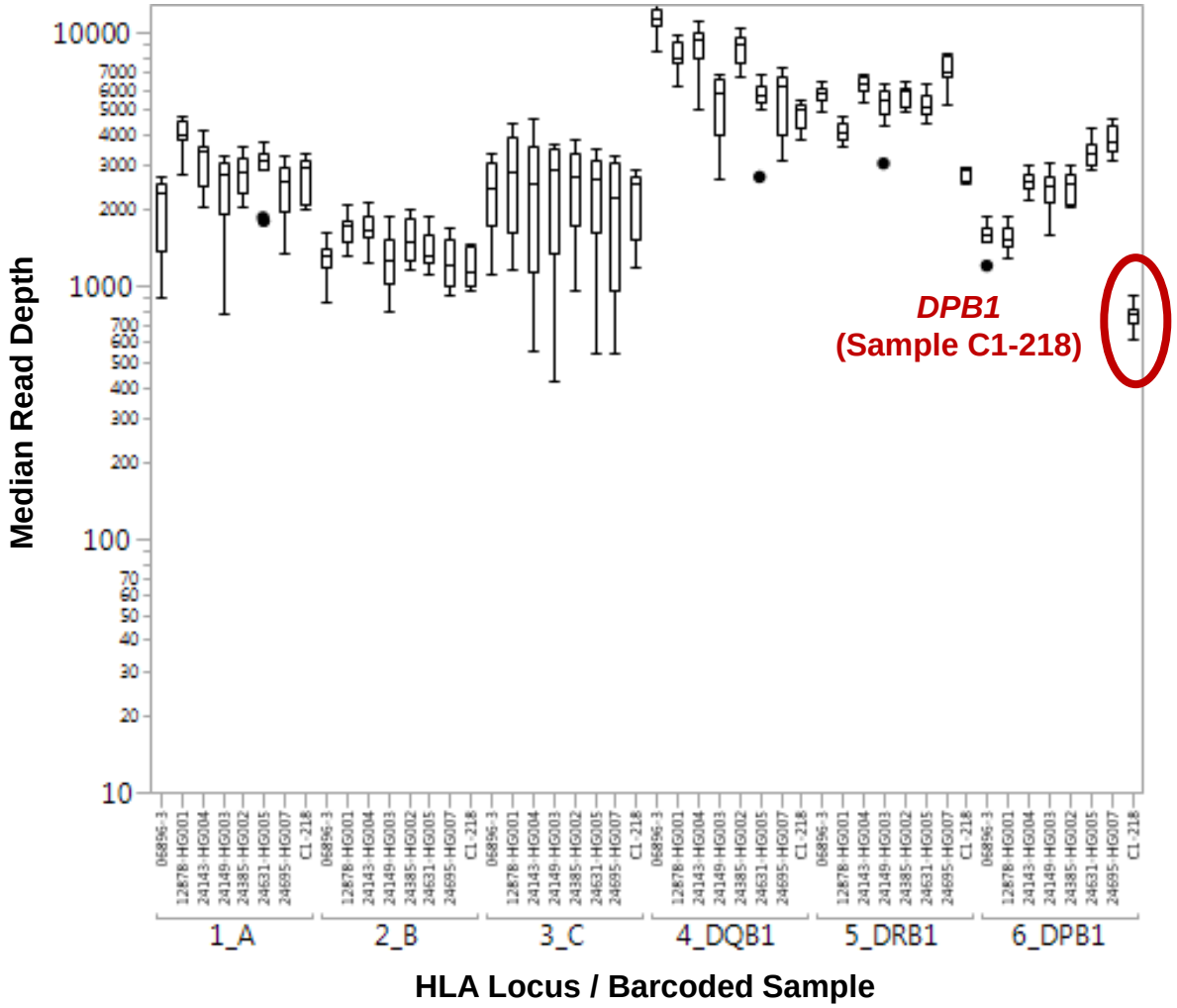
*. CCS and barcode demultiplexing analyses were performed with SMRT Link v9.0.



Sequel II System Chemistry 2.0; 54 pM on-plate loading concentration; 20-hour movie collection time; 1.1-hour pre-extension time

EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

D. HLA Read Depth Analysis Using GenDX NGSengine (Sequel II System)



- HLA locus *DPB1* generated the lowest depth of coverage with sample C1-218
  - **Note:** C1-218 gDNA sample QC showed the highest degree of DNA fragmentation with a size distribution mode of 22 kb, whereas other gDNA samples included in this dataset showed a starting modal size range from ~27 – 147 kb.
  - *DPB1* is the longest HLA amplicon (5.7 kb) amplified using the GenDX NGSgo-MX1 kit.

To help ensure adequate read coverage, particularly for DPB1 alleles, **we recommend pooling a maximum of 96 samples per Sequel II System SMRT Cell 8M** (and a maximum of 46 samples per Sequel System SMRT Cell 1M) for HLA sequencing

EXAMPLE WORKFLOW: 96-PLEX HLA AMPLICON LIBRARY PREPARATION USING GenDX NGSgo-MX6-1 KIT AND PACBIO-BARCODED OVERHANG ADAPTERS (CONT.)

D. HLA Typing Results Using GenDx NGSengine Software

Sample: **demultiplex.24143-HG004**

Full typing result

	Allele 1	*Allele 2*	*CWD 1*	*CWD 2*	*Review status*
HLA-A	**01:01:01:01**	**33:01:01:01**	C	C	Not reviewed
HLA-B	**14:02:01:01**	**35:08:01:01**	C	C	Not reviewed
HLA-C	**04:01:01:06**	**08:02:01:01**	No	C	Not reviewed
DRB1	**04:04:01**	**10:01:01:01**	C	C	Not reviewed
DQB1	**04:02:01:06**	**05:01:01:05**	No	No	Not reviewed
DPB1	**04:01:01:01**	**04:01:01:01**	C	C	Not reviewed

Example HLA typing report for 24143 (HG004) sample analyzed with GenDX NGSengine.

An **example demultiplexed HLA dataset analyzed using NGSengine** is available for download from PacBio (Link)

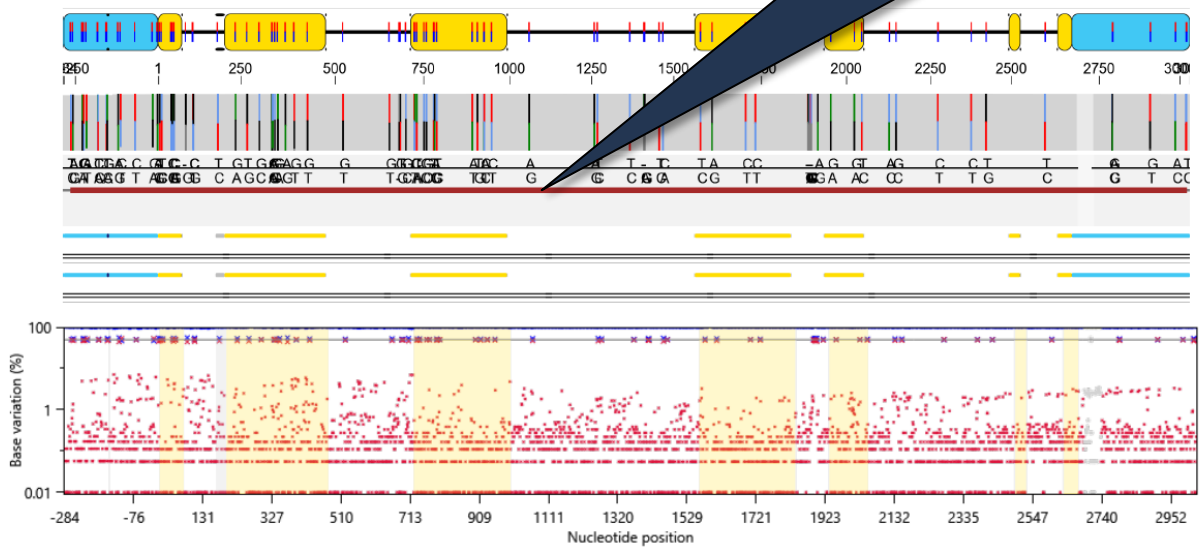
Sample: **demultiplex.24143-HG004**
Locus: **HLA-B**

Full typing result

Allele 1	*Allele 2*	*CWD 1*	*CWD 2*	*Review status*
B*14:02:01:01	**B*35:08:01:01**	C	C	Not reviewed
Core+ mm: 0 Exon+ mm: 0 Intron mm: 0	Core+ mm: 0 Exon+ mm: 0 Intron mm: 0			

Visualization

Continuous red line marking indicates the HLA data are **fully phased**



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