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

Before You Begin

The procedure describes a workflow for constructing symmetrically barcoded SMRTbell libraries from PCR products using barcoded SMRTbell adapters. In this workflow, a barcode is introduced to each amplicon through ligation with a hairpin adapter containing a unique barcode. Through this procedure, the same barcode sequence is incorporated at both ends of amplicon samples. Once barcoded, amplicons can be pooled and purified for sequencing on the Sequel®, Sequel II and Sequel IIe Systems.

Figure 1 below summarizes the multiplexed amplicon library preparation workflow using Barcoded Overhang Adapters.

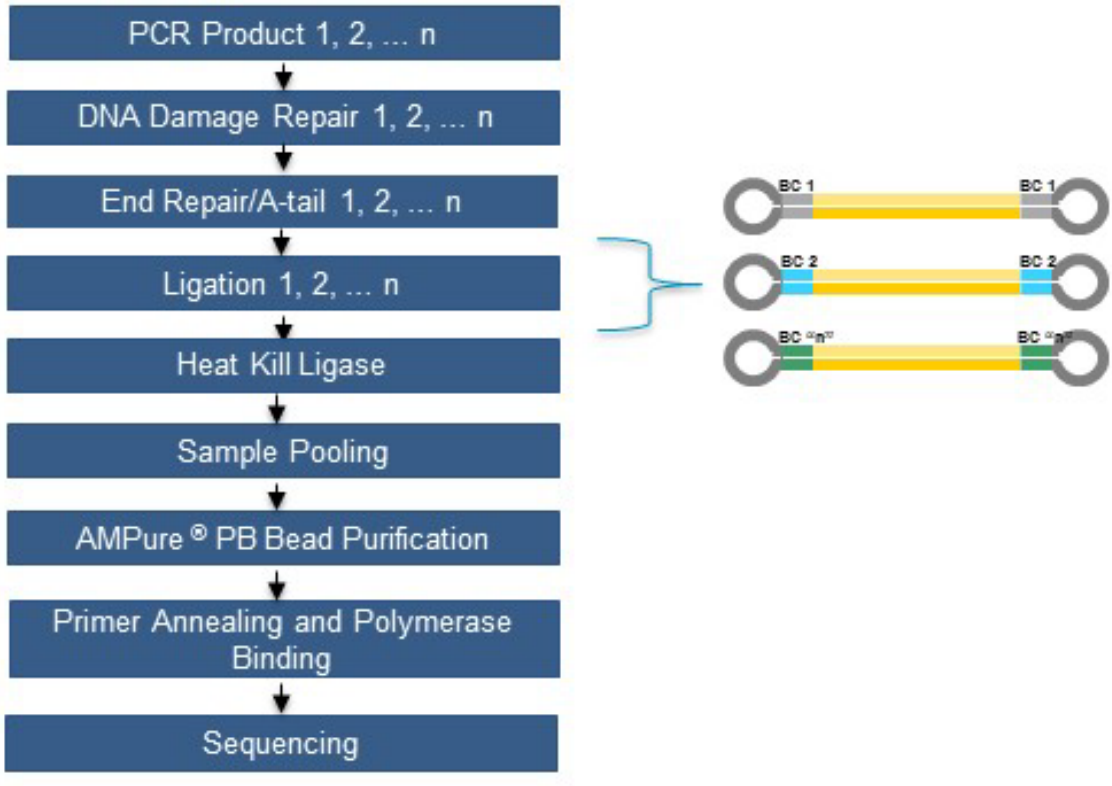

Figure 1: Barcoded overhang adapters are incorporated into PCR amplicon samples through ligation during SMRTbell library construction. Each barcoded amplicon sample contains the same barcode sequence on both ends.

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Required Materials

Item	Vendor	Part Number
Sequel® II/IIe System: SMRTbell Barcoded Adapter Plate 3.0 Sequel System: Barcoded Overhang Adapter Kit 8A or 8B	PacBio	102-009-200 (96 barcodes) 101-628-400 (8) & 101-628-500 (8)
SMRTbell® Express Template Prep Kit 2.0	PacBio	100-938-900
AMPure® PB Kit	PacBio	100-265-900
8- or 12-Multi-channel Pipette	Any MLS	Vendor-specific
PCR 8-tube strips	Any MLS	Vendor-specific
96 well plate	Any MLS	Vendor-specific

Table 1. List of Required Materials and Equipment.

Planning Multiplexing Experiments using Barcoded Overhang Adapters

Consider the following steps when planning your experiments:

1. Review the next section below 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. We recommend pooling 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 requirement per amplicon for library construction. See the “Estimating Input Requirements for Library Construction” section below.
5. Determine the appropriate concentration of AMPure PB beads to use for the purification steps. See the “Recommendations for AMPure PB Bead Purification” section below.

Best Practices for Generating High-Quality PCR Products for PacBio Sequencing

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 the presence of non-specific products, consider the following recommendations for generating high-quality amplicons suitable for SMRTbell library preparation and sequencing.

1. Begin with high-quality nucleic acids and work in a clean environment.
   a. If extracted nucleic acids must be stored, freeze at high concentrations in appropriately-buffered solutions.
   b. To minimize possible contamination and degradation caused by multiple freeze/thaw cycles, sub-aliquot DNA into smaller volumes for storage.
   c. 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.
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 oligos; 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. For mixed samples with similar targets, it is important to complete extension at every step to avoid generating chimeric products in subsequent steps. As a general guideline, use extension times of one minute per 1000 base pairs (e.g., 3 minutes for a 3 kb product).

3. Use the lowest number of cycles required for obtaining adequate yields (ng) of PCR products to proceed with SMRTbell library construction. Avoid over-amplification.
4. If non-specific products are present, optimize PCR conditions or perform AMPure PB bead-based size selection to enrich for PCR amplicons with the desired target size (see recommendations below).

Estimating Input Requirements for Library Construction

Refer to Table 2 below for DNA input requirements for library construction.

1. The values in the "Minimum Input per Amplicon into DNA Damage Repair" column are the recommended minimum DNA input amounts (ng) required for each amplicon sample to go into the first enzymatic reaction step (DNA Damage Repair).

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

Table 2. Input DNA Mass Required Per Amplicon for Multiplexing using Barcoded Overhang Adapters.

2. The values shown in the "Target DNA Mass (ng) After Pooling" column refer to the recommended minimum amount (ng) of SMRTbell library after pooling of ligation products.
3. The volume of amplicon DNA sample required for the DNA Damage Repair reaction step is 5 µL. Concentrate the sample with AMPure PB beads or dilute in 1X Elution Buffer, as necessary.

Recommendations for AMPure PB Bead Purifications

When using this procedure, it is highly recommended to purify amplicon samples before SMRTbell library construction to remove PCR reagents, buffers, primer dimers and short non-specific PCR products. Depending on the size of the target amplicon, the concentration of AMPure PB beads required for purification varies. Refer to table 3 below for the appropriate concentration of AMPure PB beads to use for purification.

Amplicon Size	AMPure PB Bead Ratio
250-500 bp	1.8X
500-1000 bp	1.0X
1-3 kb	0.60X
3-10 kb	0.45X

Table 3. Recommended AMPure PB Bead Concentration Recommendations Based on Amplicon Size.

Using a Multi-Channel Pipette

When working with a large number of reactions, we 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 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 (e.g., 1/8th of the master mix volume to each of the eight wells 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.

Reagent Handling

Several reagents that are used in the procedure (see Table 4) are sensitive to temperature and vortexing. We highly recommend that you:

- Never leave reagents at room temperature.
- Work on ice at all times when preparing master mixes.
- Finger tap followed by a quick spin prior to use.

Reagent	Where Used
DNA Damage Repair Mix v2	DNA Damage Repair
End Prep Mix	End-Repair/A-tailing
Barcoded Overhang Adapters	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation

Table 4: Temperature Sensitive Reagents.

Concentration of PCR Products Using AMPure PB Beads (If necessary)

It is highly recommended to purify each amplicon sample before library construction to remove PCR reagents, buffers, or primer dimers.

STEP	✓	Concentrate DNA	Notes
1		Add the appropriate volume of AMPure PB beads to the amplicons in clean a Lo-Bind tube. See Table 3 for recommended AMPure PB bead concentrations. The required volume depends on insert size. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Pipette mix 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate the mix on bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the beads. If the DNA is not recovered at the end of this procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube or plate from the magnetic rack. – If using tubes, use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). – If using tube strips, use ~200 µL of 80% ethanol. – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat step 8.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack and allow the beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	

12		The **minimum** volume required for elution is 6 µL. Use 1 µL for sample QC (step 14 below) and 5 µL for the first enzymatic reaction (DNA Damage Repair). For each amplicon sample to be processed, the 5 µL aliquot must contain the recommended amount of DNA listed in Table 2. If you suspect that the PCR product is too concentrated, elute in a higher volume of Elution Buffer.	
13		Add the Elution Buffer to the beads. Pipette mix 15 times. It is important to mix well. – Place at 37°C for 15 minutes to elute the DNA from the beads. – Spin the tube down, then place the tube back on the magnetic rack. – Let beads separate fully. Then without disturbing the beads, transfer the supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
14		Verify DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for sizing QC by a Bioanalyzer System.	
15		Actual recovery per µL and total available sample material: ____	

SMRTbell Library Construction

In this procedure, each amplicon sample goes through DNA Damage Repair, End-repair/A-tail and ligation with a specific barcoded overhang adapter. The required DNA input per sample for SMRTbell library construction depends on the amplicon size. Refer to Table 2 to estimate the amount of DNA required for your specific amplicon samples. Prepare master mixes according to the instructions in the DNA Damage Repair, End-Repair/A-tailing and Adapter Ligation sections below, or use the Express Amplicon Master Mix Calculator.

DNA Damage Repair

The volume of purified amplicon template material required for the DNA Damage Repair reaction is 5.0 µL. This volume must contain the required input DNA mass per sample (see Table 2). Prepare the DNA Prep Master Mix below and pipet 2.5 µL into each PCR tube or sample plate well. For convenience, the Express Amplicon Master Mix Calculator can also be used for the preparation of the master mix solution.

1. Prepare the DNA Prep Master Mix according to the table below. Note that the master mix volumes in the table below include a 25% overage.

Reagent	Tube Cap Color	For 1 rxn	1-8 Plex	12 Plex	16 Plex	24 Plex	48 Plex	96 Plex
DNA Prep Buffer		0.75	7.5	11.3	15.0	22.5	45.0	90.0
Nuclease-free Water		1.36	13.6	20.4	27.2	40.8	81.6	163.2
NAD		0.13	1.3	2.0	2.6	3.9	7.8	15.6
DNA Damage Repair Mix v2		0.26	2.6	3.9	5.2	7.8	15.6	31.2
Total Volume		2.50	25.0	37.5	50.0	75.0	150.0	300.0

Note: Do not pipet sub-microliter volumes. For <8 amplicons, prepare the "1-8-Plex" Master Mix to avoid sub-microliter pipetting.

2. Using a multi-channel pipette, transfer **2.5 µL** of DNA Prep Master Mix into a 96-well plate or PCR tubes.
3. Add **5.0 µL** of purified PCR product for a total of 7.5 µL.
4. Pipette mix 10 times. It is important to mix well.
5. Spin down the reaction mixture contents with a quick spin.
6. Incubate at 37°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

End-Repair/A-tailing

1. Prepare the End-Repair/A-tailing Master Mix according to the table below. Note that the master mix volumes in the table below include a 25% overage.

Reagent	Tube Cap Color	For 1 rxn	1-8 Plex	12 Plex	16 Plex	24 Plex	48 Plex	96 Plex
DNA Prep Buffer (µL)		0.25	2.5	3.8	5.0	7.5	15.0	30.0
Nuclease-free Water		1.75	17.5	26.3	35.0	52.5	105.0	210.0
End Prep Mix (µL)		0.50	5.0	7.5	10.0	15.0	30.0	60.0
Total Volume		2.50	25.0	37.5	50.0	75.0	150.0	300.0

Note: Do not pipet sub-microliter volumes. For <8 amplicons, prepare the "1-8-Plex" Master Mix to avoid sub-microliter pipetting.

2. Using a multi-channel pipette, add **2.5 µL** of End-Repair/A-tailing Master Mix to the DNA Damage Repair reactions for a total of 10.0 µL.
3. Pipette mix 10 times. It is important to mix well.
4. Spin down the reaction mixture contents with a quick spin.
5. Incubate at 20°C for 30 minutes.
6. Incubate at 65°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

Adapter Ligation

1. Prepare the Adapter Ligation Master Mix (without the barcoded adapter) according to the table below. Note that the master mix volumes in the table below include a 10% overage.

Reagent	Tube Cap Color	For 1 rxn	1-8 Plex	12 Plex	16 Plex	24 Plex	48 Plex	96 Plex
Ligation Mix (µL)		5.00	44.0	66.0	88.0	132.0	264.0	528.0
Ligation Additive (µL)		0.17	1.5	2.2	2.9	4.4	8.8	17.6
Ligation Enhancer (µL)		0.17	1.5	2.2	2.9	4.4	8.8	17.6
Total Volume		5.30	46.9	70.4	93.9	140.8	281.6	563.2

Note: Do not pipet sub-microliter volumes. For <8 amplicons, prepare the "1-8-Plex" Master Mix to avoid sub-microliter pipetting.

2. Using a multi-channel pipette, add **1.0 µL** of appropriate Barcoded Overhang Adapters to the End-Repair/A-tailing reactions for a total of 11.0 µL per reaction.
3. Using a multi-channel pipette, Add **5.3 µL** of Adapter Ligation Master Mix to each well or tube for a total of 16.3 µL.
4. Pipette mix 10 times. It is important to mix well.
5. Spin down the reaction mixture contents with a quick spin.
6. Incubate at 20°C for 60 minutes.
7. **IMPORTANT**: Heat Kill the ligase before proceeding to the sample pooling step. To heat kill ligase, incubate the sample at 65°C for 10 minutes, then return the reaction to 4°C.
8. Proceed to Pooling.

Pooling

After ligation with the barcoded overhang adapters, ligation reactions may be pooled for AMPure PB bead purification. Ensure that the pooled sample contains the minimum total mass (ng) of SMRTbell library as indicated in the 'Target DNA mass (ng) after pooling' column in Table 2.

1. Pool all Adapter Ligation 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.

Purification of SMRTbell Templates

For AMPure PB bead concentration guidelines, refer to Table 3.

STEP	✓	AMPure PB Bead Purification 1	Notes
1		Add the appropriate volume of AMPure PB beads to the pooled amplicons. See Table 3 for recommended AMPure PB bead concentrations. The required volume depends on insert size. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate samples on bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for a 1.5 mL DNA LoBind tube). – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **100 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	

STEP	✓	AMPure PB Bead Purification 2	Notes
1		Add the appropriate volume of AMPure PB beads to the pooled amplicons. See Table 3 for recommended AMPure PB bead concentrations. The required volume depends on insert size. Note that the beads must be brought to room temperature before use and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times. It is important to mix well.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Incubate samples on bench top for 5 minutes at room temperature.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
8		Wash beads with freshly prepared 80% ethanol. Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube (1.5 mL for a 1.5 mL DNA LoBind tube) – Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the beads. – After 30 seconds, pipette and discard the 80% ethanol.	
9		Repeat **step 8**.	
10		Remove residual 80% ethanol. – Remove tube from magnetic rack and spin. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack and allow beads to separate. – Pipette off any remaining 80% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Immediately add **50 µL** of Elution Buffer volume to your beads. Pipette mix 15 times. It is important to mix well. – Elute the DNA by letting the mix incubate at 37 ºC for 15 minutes. This is important to maximize recovery of high molecular weight DNA. – Spin the tube down, then place the tube back on the magnetic rack. – Let beads separate fully. Then without disturbing the beads, transfer supernatant to a new 1.5 ml Lo-Bind tube. – Discard the beads.	
13		Verify DNA amount and concentration using a Qubit quantitation platform. – Measure the DNA concentration using a Qubit fluorometer. – Using 1 µL of the eluted sample, make a 1:10 dilution in EB. – Use 1 µL of this 1:10 dilution to measure the DNA concentration using a Qubit fluorometer and the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for sizing QC by a Bioanalyzer System.	
14		Actual recovered DNA SMRTbell concentration (ng/µl): _____ Total recovered DNA SMRTbell amount (ng): _____	

Prepare for Sequencing

Follow the instructions in SMRT® Link Sample Setup v10.1 or higher. Select “<3kb Amplicons” or “≥3kb Amplicons” from the application dropdown as appropriate.

Sequencing Data Analysis

SMRT Link v9.0 or higher supports demultiplexing of up to 10,000 barcodes in a multiplexed data set. PacBio highly recommends upgrading to SMRT Link v9.0 or higher for performing demultiplexing analysis of pooled samples. Refer to our Multiplexing Resources website to download the appropriate barcode sequence FASTA file to use for de-multiplexing SMRT sequencing data.

Revision History (Description)	Version	Date
Initial release.	01	June 2019
Updated to include reference to 96 barcoded overhang adapter sequences and annealing procedure.	02	July 2019
Changed Binding Time for Sequel II System from 4 hours to 1 hour. Updated Sequencing instructions for Sequel and Sequel II Systems.	03	April 2020
Added “Sequel II System” to first paragraph.	04	April 2020
Updated required materials table on page 2.	05	June 2020
Updated “Prepare for Sequencing” section.	06	January 2021
Updated to list SMRTbell Barcoded Adapter Plate 3.0.	07	November 2021