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.

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

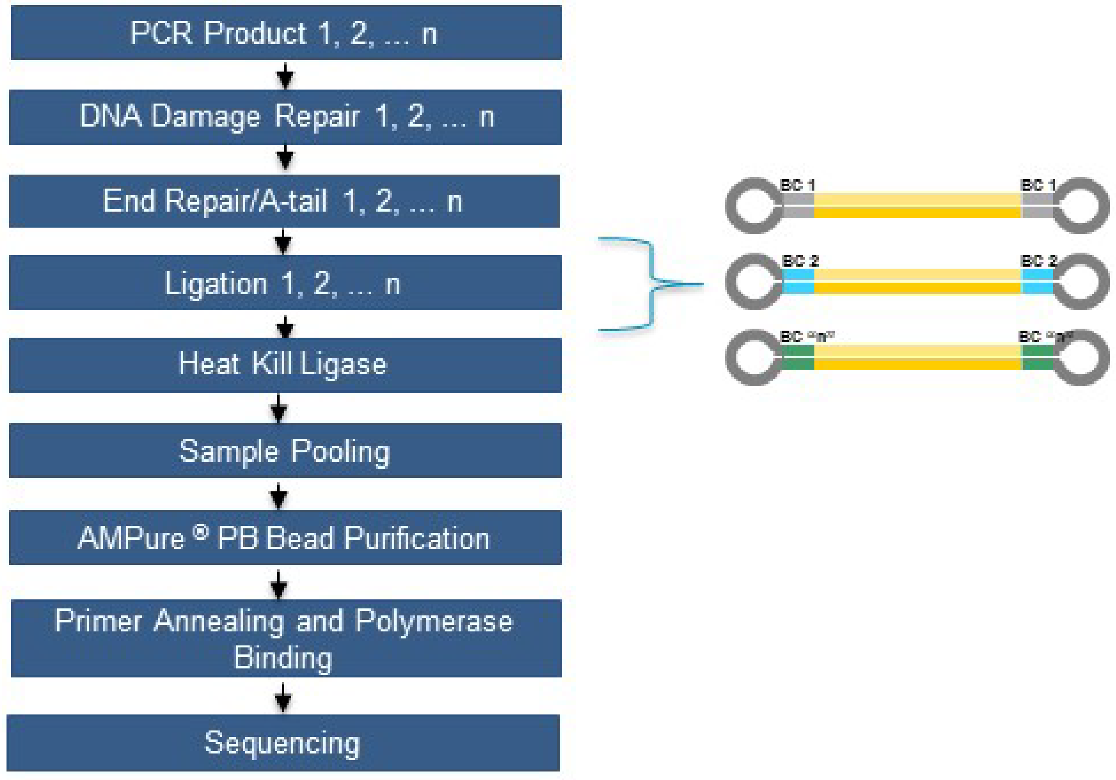

Figure 1: Barcoded overhang adapters are incorporated into the PCR amplicon through ligation during SMRTbell library construction.

Part Number 101-791-700 version 02 (July 2019)

Required Materials

Item	Where Used	Vendor	Part Number
Barcoded Overhang Adapters (see below, "Barcoded Overhang Adapters")	Library Prep	PacBio	101-629-000
SMRTbell Express Template Prep Kit 2.0	Library Prep	PacBio	100-938-900
AMPure® PB Kit	Purification	PacBio	100-265-900
8- or 12-Multi-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

Table 1. List of Required Materials and Equipment.

Barcoded Overhang Adapters

To perform this procedure, barcoded overhang adapters are required. There are 16 barcoded overhang adapters that are commercially available from PacBio: PacBio Barcoded Overhang Adapter Kit 8A, Part #101-628-400 and PacBio Barcoded Overhang Adapter Kit 8B, Part #101-628-500. We recommend both sets for multiplexing up to 16 samples.

To multiplex up to 96 samples, we offer an annealing protocol and a list of barcoded overhang adapter sequences to order from an oligo vendor provider. Please contact your Pacific Biosciences for updates on commercially available products.

Planning Multiplexing Experiments using Barcoded Overhang Adapters

Consider the following steps when planning 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. 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 requirement per amplicon for library construction. See the "Estimating input requirements for library construction" section below.
5. Finally, determine the appropriate concentration of AMPure PB beads to use for the purification steps. See the "Recommendations for using AMPure PB beads 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. For DNA samples, DNAStable® Plus from Biomatrica may be used to help preserve extracted DNA.
   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 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. 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 numbers in the "Required Mass per Amplicon into DNA Damage Repair" column are the recommended amplicon masses required for the first enzymatic reaction step (DNA Damage Repair), regardless of the number of amplicons to be multiplexed.

Amplicon Size (bp)	Required Mass per Amplicon into DNA Damage Repair (ng)
250	10
500	10
1000	50
3000	50
5000	100
10000	150
15000	200

Table 2. DNA Required Per Amplicon for Multiplexing.

2. The volume of the sample required for DNA Damage Repair is 5 µL. Concentrate the sample with AMPure PB, or dilute in 1x Elution Buffer, as necessary.

Recommendations for AMPure PB Bead Purifications

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 bead for purification varies. Use the table below to determine the appropriate concentration of AMPure PB beads.

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

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

Using a Multi-Channel Pipette

When working with a large number of reactions, we 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 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.

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

It is highly recommended to purify amplicons 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. 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 bead 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 bead 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 bead 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. 1 µL for sample QC and 5 µL for the first enzymatic reaction (DNA Damage Repair). The 5 µL aliquot must contain the amount of DNA required listed on Table 2. If you suspect that the PCR product is concentrated, elute in higher volume.	
13		Add the Elution Buffer volume to your 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 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.	
14		Verify your 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 dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for QC by Bioanalyzer.	
15		Actual recovery per µL and total available sample material: ____	

Reagent Handling

Several tubes in the kits (shown in Table 4 below) are sensitive to temperature and vortexing. We highly recommend that you:

- Never leave tubes 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
Overhang Adapter v3	Ligation
Ligation Mix	Ligation
Ligation Additive	Ligation
Ligation Enhancer	Ligation

Table 4: Temperature Sensitive Reagents.

Annealing PacBio SMRTbell Overhang Adapters

Prepare 10X Annealing Buffer:

1. Prepare 10X Annealing Buffer (Formulation: 100 mM Tris-HCl, 1 M NaCl, pH 7.5)

Resuspend lyophilized oligos:

1. Spin the tubes for 10 seconds at maximum speed.
2. Resuspend oligos in nuclease-free 10 mM Tris HCl pH 7.5 to final concentration of 170 µM.
3. Cap and vortex for 1 minute to ensure everything is dissolved.
4. Place the resuspended oligo on ice for 30 minutes prior to use.

Prepare PacBio Barcoded Overhang SMRTbell Adapters for Annealing:

1. Prepare 17 µM working stock using 10X Annealing Buffer and nuclease-free water (molecular biology grade). Final concentration of Annealing Buffer is 1X.
2. Measure the final concentration using UV/Vis Nanodrop of Qubit.

   Note: Accuracy of the final concentration can be improved by targeting a higher initial working stock concentration (10-20%) and adjusting down to the final concentration using the measured concentration of the initial working stock.

Anneal PacBio Barcoded Overhang SMRTbell Adapters:

1. Anneal the 17 µM working stock as follows:
   - Incubate in a thermal cycler at 95°C for 5 minutes,
   - then transfer the tubes or plates at room temperature (25° C) for 30 min.
   - Hold at 4° C.
2. Spin down tubes or plates at maximum speed for 1 minute.
3. Store all annealed adapter preparations at -15 to -25° C

   Note: Annealed working stock can be prepared in advance and stored without requiring additional reannealing. Store at -20° C for long term storage.

SMRTbell Library Construction

The required input DNA per sample depends on the amplicon size and number of amplicons to be multiplexed. Refer to Table 2 to estimate the amount of DNA required for your amplicons.

DNA Damage Repair

The volume of template required for the DNA Damage Repair reaction is 5.0 µL. This volume must contain the calculated mass required for each sample for multiplexing. Prepare the master mix below and pipet 2.5 µL into each PCR tube or sample plate well. The Express Amplicon Master Mix Calculator can also be used in the preparation of the master mix for your convenience.

1. Prepare DNA Prep Master Mix according to the table below. Note that the master mix volumes in the table below include 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, we recommend preparing the 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 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 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, we recommend preparing the 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 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, we recommend preparing the 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 the 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 adapters, samples can be pooled for AMPure PB bead purification.

1. 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.

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 bead 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 **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 bead 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 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.	
13		Verify your 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 dsDNA HS Assay kit according to the manufacturer's recommendations. The remaining 9 µL of 1:10 diluted sample may be used for QC by BioAnalyzer.	
14		Actual recovered DNA SMRTbell concentration (ng/µl): __________________ Total recovered DNA SMRTbell amount (ng): ______________________	

Anneal and Bind SMRTbell Library Templates

Use SMRT Link Sample Setup for instructions for primer annealing and polymerase binding.
For primer annealing, use **Sequencing Primer v4** (found in the SMRTbell Express Template Prep Kit 2.0). Sequencing Primer v4 is for diffusion loading **only** and cannot be used for MagBead loading.

Prepare for Sequencing

For the Sequel System, Diffusion loading is recommended for loading amplicon libraries prepared by the SMRTbell Express Template Prep Kit 2.0.

Follow the Sample Setup in SMRT Link for preparing your sample for sequencing.

For detailed recommendations for sequencing of specific library insert size ranges, refer to the Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System here.

Demultiplex barcoded Samples

To demultiplex barcoded samples we recommend using the following support fasta file: Sequel_96_barcodes_v2.

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

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