

Procedure & Checklist – Preparing Multiplexed Microbial Libraries Using SMRTbell® Express Template Prep Kit 2.0

Before You Begin

This procedure describes how to prepare multiplexed SMRTbell libraries for sequencing on the Sequel and Sequel II Systems. For each microbe, 1 µg of gDNA is required. For optimal results, input gDNA must migrate predominantly above 20 kb as determined by pulsed-field gel electrophoresis. To ensure even coverage across the genome, be sure to isolate gDNA from bacterial cultures which have reached stationary growth phase.

This procedure includes instructions for multiplexing a minimum of 2 to a maximum of 16 genomes (up to a total sum of 30 MB of genome sizes including plasmid elements) on the Sequel System. For the Sequel II System, PacBio recommends multiplexing a minimum of 32 to a maximum of 48 genomes (up to 200 Mb).

Options for size-selection using AMPure PB Beads and BluePippin are also provided. BluePippin size-selection may improve microbial genome assembly of microbes with long repeat regions of >6 kb. For this use-case, the average pooled library insert size should be approximately 12.5 kb - 15 kb.

A [Microbial Multiplexing Calculator](#) is also available to calculate volumes needed to attain equimolar pooling as well as determining master mix volumes for SMRTbell library construction. Note: If you have issues accessing this Excel spreadsheet through the Chrome browser, we recommend trying Internet Explorer, Safari or Firefox. If you continue to have access issues, please contact your local Field Application Scientist.

Required Materials

Item	Vendor	Part Number
DNA QC (One of the following)		
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA or	Bio-Rad	170-3670
Pippin Pulse Electrophoresis Power Supply or	Sage Science	PP10200
Femto Pulse®	Agilent	P-0003-0817
2100 Bioanalyzer Instrument (sheared DNA only)	Agilent	G2939BA
DNA Quantitation		
Qubit™ Fluorometer	ThermoFisher Scientific	Q33226
Qubit™ 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q33230
DNA Shearing (One of the following)		
g-TUBE	Covaris	10145
Megaruptor	Diagenode	B06010001
Long Hydropores	Diagenode	E07010002
Hydrotubes	Diagenode	C30010018
Megaruptor 3	Diagenode	B06010003
Megaruptor 3 Shearing Kit	Diagenode	E07010003
SMRTbell Library Preparation		
SMRTbell Express Template Prep Kit 2.0	PacBio	100-938-900
Barcoded Overhang Adapter Kit 8A (8 adapters)	PacBio	101-628-400
Barcoded Overhang Adapter Kit 8B (8 adapters)	PacBio	101-628-500
96 Barcoded Overhang Adapters*	Any Oligo Synthesis Company	
Eppendorf MiniSpin Plus or other equivalent benchtop centrifuge model	Eppendorf	022620100
Magnetic Bead Rack	ThermoFisher Scientific	12321D
0.2 mL PCR 8-strip tubes	USA Scientific	1402-4708
Wide Orifice Tips (Tips LTS W-O 200 UL Fltr RT-L200WFLR)	Rainin	17014294
AMPure® PB Beads	PacBio	100-265-900
Elution Buffer	PacBio	101-633-500
100% Ethanol, Molecular Biology Grade	Any MLS	
Thermomixer	Any MLS	
Vortex mixer	Any MLS	
96-well PCR Plate	Eppendorf	951020303
96-well PCR Plate Seal	BioRad ThermoFisher Scientific	MSB1001 4306311
Multi-channel Pipettes	Rainin	17013810 17013808 17013807
Magnetic Plate	ThermoFisher Scientific Permagen	AM10027 T480
Size-selection (If not using AMPure PB Size Selection)		
BluePippin Size-Selection System	Sage Science	BLU0001
BluePippin with dye free, 0.75% Agarose Cassettes and S1 Marker	Sage Science	BLF7510

*See "Barcoded Overhang Adapters" section below.

Table 1. List of Required Materials and Equipment.

Barcoded Overhang Adapters

To perform this procedure, use barcoded overhang adapters. For the Sequel System, PacBio Barcoded Overhang Adapter Kit 8A or 8B are available for multiplexing up to 16 microbes.

For the Sequel II System, an annealing protocol is provided and a list of 48 barcoded overhang adapters is available in Appendix 2. The recommended 48 barcodes and their sequences are included in the oligo ordering sheet found [here](#). Barcoded overhang adapters may be ordered from your favorite oligo synthesis company (e.g. IDT).

Workflow

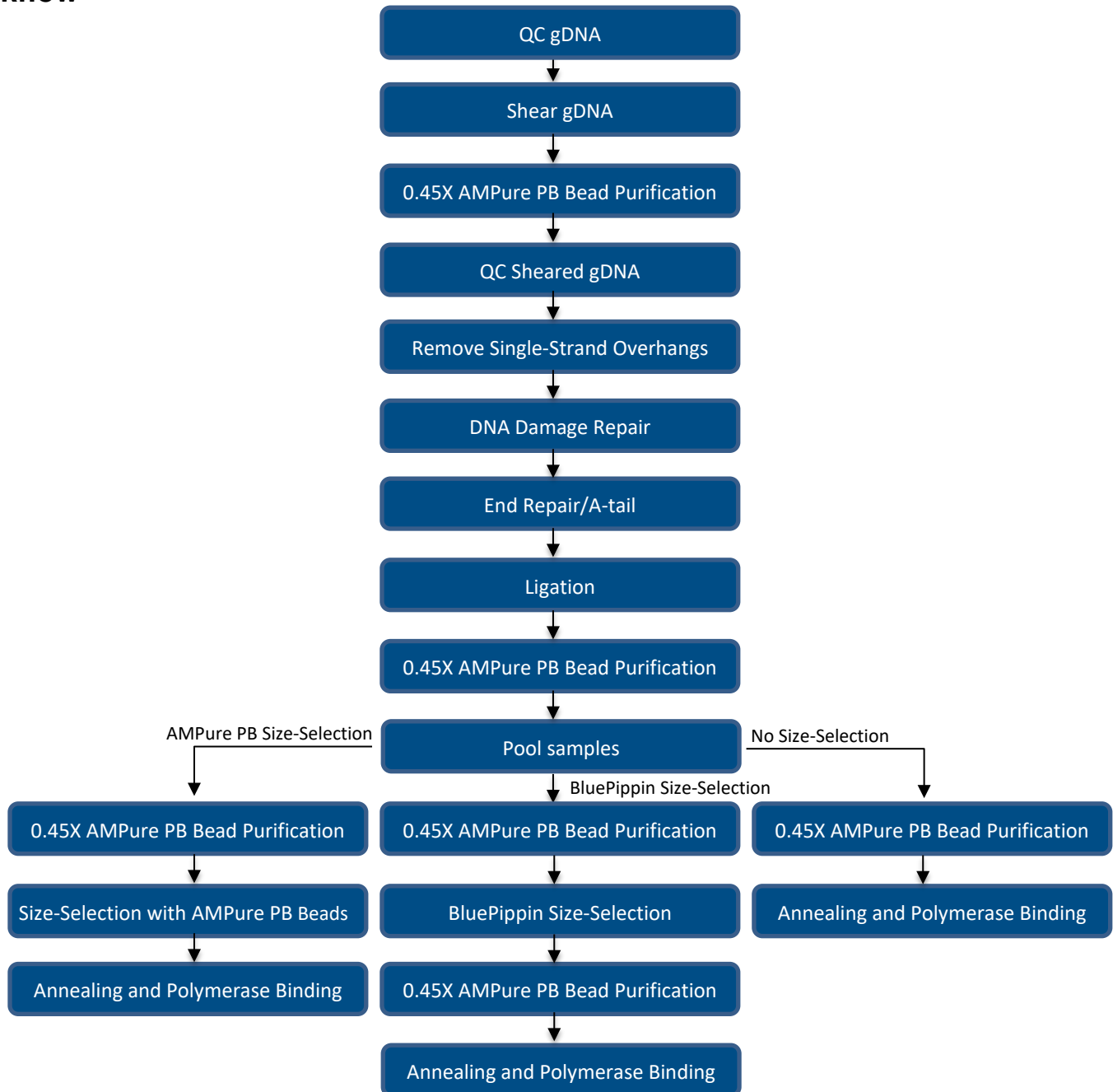


Figure 1: Workflow for preparing multiplexed microbial library using the SMRTbell Express Template Prep Kit 2.0 and Barcoded Overhang Adapters.

Best Practices Recommendations

- When working with a large number of reactions, we highly recommend using a multi-channel pipette along with a 96-well plate or 8-tube strips.
- This procedure requires accurate pipetting of small volumes. For pipetting $\leq 2 \mu\text{L}$, use a P2 pipette or a multi-channel pipette that has been recently calibrated and is in good working condition.
- For mixing the reactions in a 96-well plate, seal the plate and pulse-vortex followed by a quick spin-down. Mixing with a multichannel pipette using wide-bore pipette tips can also be performed. However, there may be some sample loss due to samples sticking to the wide-bore tips.
- Ensure that the AMPure PB Beads are at room temperature prior to performing the purification steps.
- For 2- and 3-plexes on the Sequel System, we recommend starting with twice the DNA input amount recommended above (i.e., use $2.0 \mu\text{g}$ of input gDNA for 2- and 3-plexes to ensure sufficient quantity of final pooled SMRTbell library for sequencing). Throughout this procedure, use double the standard volumes of input DNA and master mix reagents at each step for 2- and 3-plexes, as indicated in Appendix 1.

gDNA Quantification and Qualification

Measure gDNA concentration using a Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer.

Assess gDNA size and integrity by pulsed field gel electrophoresis (PFGE) or an equivalent method before beginning library preparation. Any of the three commercially available systems listed in Table 2 below may be used to evaluate gDNA quality. Links to recommended procedures for each are also provided in the table. Suitable input genomic DNA should migrate predominantly above 20 kb.

Method	Procedure
Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis System	Procedure & Checklist - Using the BIO-RAD CHEF Mapper XA Pulsed Field Electrophoresis System
Agilent Technologies, Inc. Femto Pulse	Agilent Technologies, Inc.
Sage Science Pippin Pulse	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

Table 2. gDNA Quality QC Methods and Procedures.

Shear Genomic DNA Using a Covaris g-TUBE

Always perform test shears to determine the best parameters to meet the above size distribution mode requirements. Additionally, the response of individual gDNA samples to shearing parameters may differ, so small-scale test shears are always required.

STEP	✓	Shear DNA	Notes
1		Dilute 1.0 µg gDNA into 100 µL 1X Elution Buffer to a final concentration of 10 ng/µL. Before shearing, we recommend removing a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Transfer gDNA to the g-TUBE and centrifuge at 2029 x g (5500 rpm in the Eppendorf MiniSpin Plus) for 2 minutes to achieve a target mode size of 10 kb - 15 kb.	
3		Repeat spin until the entire gDNA sample has passed through the orifice. (This may take 2-3 spins.)	
4		Invert the g-TUBE and centrifuge at 2029 x g (5500 rpm in the Eppendorf MiniSpin Plus) for 2 minutes.	
5		Repeat spin until the entire gDNA sample has passed through the orifice. (This may take 2-3 spins.)	
6		Transfer the sheared gDNA to a fresh 1.5 mL Lo-bind microfuge tube.	
7		Evaluate the size distribution of the resulting sheared gDNA fragments by running the un-sheared and sheared samples on a Pulsed Field Electrophoresis system, Femto Pulse or Pippin Pulse. If DNA is over-sheared, decrease centrifugation speed. If DNA is under-sheared, increase centrifugation speed.	
8		Proceed to the "Concentrate sheared gDNA using AMPure PB Beads" section to concentrate the sample using AMPure PB beads.	

Shear Genomic DNA Using Megaruptor 1 or Megaruptor 2

Always perform test shears to determine the best parameters to meet the above size distribution mode requirements. Additionally, the response of individual gDNA samples to shearing parameters may differ, so small-scale test shears are always required. For test shears, use the recommended concentration and volume below.

	✓	Shear DNA	Notes
1		Dilute gDNA to 1 ng/µL - 50 ng/µL in Elution Buffer. The sample volume may range from 50 µL - 400 µL . It is important not to exceed this DNA concentration during shearing or the hydropore may become clogged. Before shearing, we recommend removing a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Shear gDNA using long hydropores and software setting of 10 kb or 15 kb (based on what you have determined as optimal during small-scale test shears to achieve a target mode size of 10 kb - 15 kb).	
3		Evaluate the size distribution of the resulting sheared gDNA fragments by running the un-sheared and sheared samples on a Pulsed Field Electrophoresis system, Femto Pulse or Pippin Pulse. Note that the Megaruptor 1 will dilute DNA samples during shearing, so empirically measure the volume of the sheared gDNA sample before performing AMPure PB bead purification.	
4		Proceed to the "Concentrate sheared gDNA using AMPure PB Beads" section to concentrate the sample using AMPure PB beads.	

Shear Genomic DNA Using Megaruptor 3

Always perform test shears to determine the best parameters to meet the above size distribution mode requirements. Additionally, the response of individual gDNA samples to shearing parameters may differ, so small-scale test shears are always required. For test shears, use the recommended concentration and volume below.

	✓	Shear DNA	Notes
1		Dilute gDNA to 3 ng/μL in Elution Buffer in a final sample volume of 150 μL . Before shearing, we recommend removing a 1 μL aliquot (un-sheared sample) for sizing QC.	
2		Shear gDNA at a speed of 33-35 to achieve a target mode size of 10 kb - 15 kb.	
3		Evaluate the size distribution of the resulting sheared gDNA fragments by running the un-sheared and sheared samples on a Pulsed Field Electrophoresis system, Femto Pulse or Pippin Pulse. Measure the volume of the sheared gDNA sample before performing AMPure PB bead purification.	
4		Proceed to the "Concentrate sheared gDNA using AMPure PB Beads" section to concentrate the sample using AMPure PB beads.	

Concentrate sheared gDNA using AMPure PB Beads

STEP	✓	Concentrate DNA	Notes
		Notes for proper handling of AMPure PB beads: <ul style="list-style-type: none"> – Bring AMPure PB beads to room temperature before use. – AMPure PB bead purification steps should be performed at room temperature. – Before using, mix the bead reagent well until solution appears homogeneous. – Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process. 	
1		Add 0.45X volume of AMPure PB beads to each sheared gDNA sample.	
2		Pipette mix 15 times with wide-bore pipette tips. 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 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		<p>Wash beads with freshly prepared 80% ethanol.</p> <p>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.</p> <ul style="list-style-type: none"> – Do not remove the tube from the magnetic rack. – 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). 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		<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> – 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		<p>Elute purified DNA from the beads (the volume to go into “Remove Single-Strand Overhangs”).</p> <ul style="list-style-type: none"> – Add 16 µL of Elution Buffer to the beads – Pipette mix 15 times with wide-bore pipette tips. It is important to mix well. – Elute the DNA by letting the mix incubate at 37°C 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		The sheared DNA can be stored for up to 2 weeks at 4°C or at -20°C for longer duration. Do not freeze/thaw.	

Quantification and Qualification of Sheared gDNA

1. Dilute 1 μL of the purified sheared gDNA sample into 9 μL 1X Elution Buffer. Use 1 μL of this 1:10 dilution to determine DNA concentration using a Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer. Expected yield of sheared gDNA is 50-80% depending on gDNA quality.
2. Use the remaining 9 μL of the 1:10 dilution to evaluate size distribution of sheared gDNA by standard gel or field inversion agarose or capillary gel electrophoresis. The average size of the sheared gDNA should be approximately 10 kb - 15 kb. (If performing BluePippin size selection, the average pooled SMRTbell library insert size should be approximately 12.5 kb - 15 kb.)

Note: If the concentration of sheared and AMPure PB purified gDNA is $<33 \text{ ng}/\mu\text{L}$ for any microbe, choose one of the following options:

- If available, shear additional gDNA, pool sheared gDNA, and perform another AMPure PB bead purification step. Be sure to elute sheared gDNA in an appropriate volume to achieve $\geq 33 \text{ ng}/\mu\text{L}$.
- If additional gDNA is not available, normalize the concentration of all samples to the lowest sample concentration available and proceed with the instructions in this procedure. We do not recommend starting with total sum of input sheared gDNA amounts of $<1000 \text{ ng}$ for non-size-selected libraries or $<2000 \text{ ng}$ for size-selected libraries. For example, starting with $16.5 \text{ ng}/\mu\text{L}$ sheared gDNA for each microbe in an 8-plex would give a total input DNA mass of 1927 ng ($14.6 \mu\text{L} * 16.5 \text{ ng}/\mu\text{L} * 8$).
- If additional gDNA is not available and the concentration of one or more samples after shearing and AMPure PB bead purification is $<16.5 \text{ ng}/\mu\text{L}$, consider excluding that microbe from the pool, if possible.
- If none of the above options is possible, proceed with the maximum amount(s) of the limiting sample(s) available and enter the DNA sample concentration values measured in Step 13 of the 'Purify SMRTbell Library Using 0.45X AMPure® PB Beads Before Pooling' section (Page 12) into the [Microbial Multiplexing Calculator](#). Note that proceeding with this option may increase variation in relative coverage among the microbes in the pool.

Prepare SMRTbell Templates Using Express Template Prep Kit 2.0

Reagent Handling

Several tubes in the SMRTbell Express TPK 2.0 kit (shown in Table 3 below) are sensitive to temperature and vortexing. We recommend:

- 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 Prep Additive	Remove single-strand overhangs
DNA Prep Enzyme	Remove single-strand overhangs
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 3: Temperature sensitive reagents

Recommendations for Annealing Barcoded Overhang SMRTbell Adapters

For barcoded overhang adapters ordered from IDT or your oligo synthesis provider, the oligos must be annealed to form hairpins prior to use.

For annealing, the following reagents are required:

- 10X Annealing Buffer (100mM Tris-HCl, 1M NaCl, pH 7.5)
- Barcoded Overhang Adapter diluted to 17 μ M

Preparing Lyophilized Oligos:

1. Spin the tubes for 10 seconds at maximum speed.
2. Resuspend oligos in nuclease-free 10mM Tris-HCl pH 7.5 to a final concentration of 170 μ M.
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.

Preparing and Annealing Barcoded Overhang SMRTbell Adapters:

1. Prepare 17 μ M working stock using 10X Annealing Buffer and nuclease-free water (molecular biology grade).

Reagent	Volume	✓	Notes
Barcoded Overhang Adapter (170 μ M)	1.0 μ L		
10x Annealing Buffer	1.0 μ L		
Molecular Biology Grade Water	8.0 μ L		
Total Volume	10.0 μ L		

2. Measure the final concentration using UV/Vis Nanodrop or Qubit.

Note: Final concentration accuracy 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.



3. Anneal the 17 μ M working stock as follows:
 - Incubate in a thermal cycler at 95°C for 5 minutes,
 - Incubate at 25° C for 30 minutes.
 - Hold at 4°C.
4. Spin down tubes or plates at maximum speed for 1 minute.
5. 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.

Remove Single-Strand Overhangs





When working with large number of reactions, we recommend performing reactions in a 96-well plate or 8-tube strips and using a multi-channel pipette for reagent transfer.

1. Adjust sheared gDNA concentration to 33 ng/μL with 1X Elution Buffer, then aliquot **14.6 μL** of each sheared gDNA sample at 33 ng/μL into a tube.
2. The DNA Prep Additive must be diluted first for use in step 3 below. Dilute the stock DNA Prep Additive by diluting it 1:5 in Enzyme Dilution Buffer. The table below provides enough volume for up to 8-plex. Scale up the dilution volume below for higher plex reactions.

Reagent	Tube Cap Color	Volume	✓	Notes
Enzyme Dilution Buffer		4.0 μL		
DNA Prep Additive		1.0 μL		
Total Volume		5.0 μL		

The diluted DNA Prep Additive is one-time use only and should not be stored for future use.

3. Prepare DNA Prep Master Mix according to the table below (or the Master Mix Tables provided in the [Microbial Multiplexing Calculator](#)).

Reagent	Tube Cap Color	8 Plex	16 Plex	32 Plex	48 Plex	N Plex
DNA Prep Buffer		23.3	46.6	93.2	139.8	N X 2.33 X 1.25
NAD		3.3	6.6	13.2	19.8	N X 0.33 X 1.25
Diluted DNA Prep Additive (step 2 above)		3.3	6.6	13.2	19.8	N X 0.33 X 1.25
DNA Prep Enzyme		3.3	6.6	13.2	19.8	N X 0.33 X 1.25

Note that indicated volumes include 25% overage.



4. For each sample, add 3.3 μL of the DNA Prep Master Mix to 14.6 μL sheared DNA.

Reagent	Volume	✓	Notes
Sheared DNA	14.6 μL		
DNA Prep Master Mix	3.3 μL		
Total Volume	17.9 μL		

5. Finger tap to mix then very briefly spin to return contents to the bottom of the tube (for Lo-Bind microcentrifuge tubes and 8-tube strips). For 96-well plates, seal the plate and pulse vortex the plate followed by quick spin down.
6. Incubate at 37°C for 15 minutes, then return the reaction to 4°C. Proceed to the next step.

DNA Damage Repair

1. Prepare DNA Damage Master Mix according to the table below (or the Master Mix Tables provided in the [Microbial Multiplexing Calculator](#)).

Reagent	Tube Cap Color	8 Plex	16 Plex	32 Plex	48 Plex	N Plex
DNA Damage Repair Mix v2		6.7	13.4	26.8	40.2	N X 0.67 X 1.25
Enzyme Dilution Buffer		3.3	6.6	13.2	19.8	N X 0.33 X 1.25

Note that indicated volumes include 25% overage.


2. For each sample, add 1.0 µL of DNA Damage Master Mix to 17.9 µL single-strand digested sample.:

Reagent	Volume	✓	Notes
Single-strand digestion reaction	17.9 µL		
DNA Damage Master Mix	1.0 µL		
Total Volume	18.9 µL		

3. Finger tap to mix then very briefly spin to return contents to the bottom of the tube (for Lo-Bind microcentrifuge tubes and 8-tube strips). For 96-well plates, seal the plate and pulse vortex the plate followed by quick spin down.
4. Incubate at 37°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

End-Repair/A-tailing

1. For each sample, add 1.0 µL of End Prep Mix to 18.9 µL damage-repaired sample.

Reagent	Tube Cap Color	Volume	✓	Notes
Damage Repaired sample		18.9 µL		
End Prep Mix		1.0 µL		
Total Volume		19.9 µL		

2. Finger tap to mix then very briefly spin to return contents to the bottom of the tube (for Lo-Bind microcentrifuge tubes and 8-tube strips). For 96-well plates, seal the plate and pulse vortex the plate followed by quick spin down.
3. Incubate at 20°C for 10 minutes, then 65°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

Ligate Barcoded Overhang Adapters

- For ligation, the barcoded overhang adapter must be added to the A-tailed sample first before adding ligase. Add 2.0 µL of Barcoded Overhang Adapter to 19.9 µL of A-tailed sample.

Reagent	Volume	✓	Notes
A-tailed sample	19.9 µL		
Barcoded Overhang Adapter	2.0 µL		
Total Volume	21.9 µL		

- Finger tap to mix then very briefly spin to return contents to the bottom of the tube (for Lo-Bind microcentrifuge tubes and 8-tube strips). For 96-well plates, seal the plate and pulse vortex the plate followed by quick spin down.
- Let sit on ice and proceed to the next step.
- Prepare Ligation Master Mix according to the table below (or the Master Mix Tables provided in the [Microbial Multiplexing Calculator](#)).

Reagent	Tube Cap Color	8 Plex	16 Plex	32 Plex	48 Plex	N Plex
Ligation Mix		88.0	176.0	352	528	N X 10.0 X 1.10
Ligation Additive		2.9	5.8	11.6	17.4	N X 0.33 X 1.10
Ligation Enhancer		2.9	5.8	11.6	17.4	N X 0.33 X 1.10

Note that indicated volumes include 10% overage.

- For each sample, add 10.7 µL of ligation master mix to 21.9 µL of A-tailed/Barcoded Adapter mix.

Reagent	Volume	✓	Notes
A-tailed /Barcoded Adapter mix	21.9 µL		
Ligation Master Mix	10.7 µL		
Total Volume	32.6 µL		

- Finger tap to mix then very briefly spin to return contents to the bottom of the tube (for Lo-Bind microcentrifuge tubes and 8-tube strips). For 96-well plates, seal the plate and pulse vortex the plate followed by quick spin down.
- Incubate at 20°C for 60 minutes.
- Heat kill the ligase before proceeding to the sample pooling by incubating the samples at 65°C for 10 minutes.
- Proceed to the next step.

Purify SMRTbell Library Using 0.45X AMPure® PB Beads Before Pooling

For even representation across samples, equal molar pooling must be performed. It is therefore highly recommended to purify, quantify and qualify each sample for pooling. If you are purifying samples individually, use 1.5 mL tubes. If your samples are in 8-tube strips or a 96-well plate, you will need to use a magnetic separation plate for separation.

STEP	✓	AMPure PB Bead Purification	Notes
1		Adjust the volume to 100 µl with Elution Buffer and add 0.45X volume of AMPure PB	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube. For 96-well plates, seal the plate and pulse vortex the plate for mixing.	
3		Quickly spin down the tube/plate (for 1 second) to collect the beads.	
4		Incubate the mix on a bench top for 5 minutes at room temperature.	
5		Quickly spin down the tube/plate (for 1 second) to collect beads.	
6		Place the tube/plate 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/plate). Avoid disturbing the bead pellet.	
8		<p>Wash beads with freshly prepared 80% ethanol.</p> <p>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.</p> <ul style="list-style-type: none"> – Do not remove the tube/plate from the magnetic rack. – Use a sufficient volume of 80% ethanol to fill the tube/fill the wells of the 96-well plate to avoid spillage or cross contamination. . Slowly dispense the 80% ethanol against the side of the tube opposite the beads. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 80% ethanol. 	
9		Repeat step 8 .	
10		<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> – Remove tube/plate from magnetic bead rack and quickly spin to collect the beads. Both the beads and any residual 80% ethanol will be at the bottom of the tube. – Place the tube back/plate on magnetic bead rack. – Pipette off any remaining 80% ethanol. 	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10 .	
12		<p>Elute in 20 µL 1x Elution buffer.</p> <p>Finger-tap the tube to mix until beads are uniformly re-suspended (for Lo-Bind microcentrifuge tubes and 8-tube strip).</p> <p>For 96-well plate, seal the plate and pulse vortex the plate followed by quick spin down. Ensure that the beads are fully re-suspended.</p> <ul style="list-style-type: none"> – Elute the DNA by letting the mix incubate at 37°C for 15 minutes. – Quickly spin the tube or plate to collect the beads, then place the tube or plate back on the magnetic bead rack. – Let beads separate fully. Then without disturbing the bead pellet, transfer supernatant to a new 1.5 mL DNA Lo-Bind tube or new 96-well plate. – Discard the beads. 	

13	<p>Measure the DNA concentration using a Qubit fluorometer.</p> <ul style="list-style-type: none"> – Use 1 µL of the eluted sample to do a 1:5 dilution in Elution Buffer. – Use 1 µL of the diluted sample to measure the DNA concentration using a Qubit fluorometer and the dsDNA HS Assay kit according to the manufacturer's recommendations. – Enter the DNA sample concentration values into the Optional Sample Concentration column of the Pooling Calculator. – Use the remaining 4 µL of 1:5 dilution to evaluate the size distribution using a Femto Pulse system. 	
14	Proceed with sample pooling or store the sample at 4°C for future use.	

Sample Pooling

Pool samples according to the volumes provided by the [Microbial Multiplexing Calculator](#). An example of pooling volumes for a typical 10-plex is shown in Figure 2 below. The minimum recommended pooling volume is 100 µL.

The pooling volume to enter in the pooling calculator depends on the number of samples being pooled. As a rule of thumb, scale up by 50 µL for every 8-plex (starting from 100 µL). For example, use 200 µL for 32-plex, 300 µL for 48-plex.

Multiplex Level	Total Pool Volume (µL)
8	100
16	150
32	200
40	250
48	300

If unequal amounts of sheared DNA, or less than 500 ng of sheared DNA for any sample, were input into the DNA Prep Master Mix reaction to remove single-stranded overhangs, be sure to also enter the DNA sample concentration values measured in Step 13 of the 'Purify SMRTbell Library Using 0.45X AMPure® PB Beads Before Pooling' section (Page 12) into the Optional Sample Concentration column of the Pooling Calculator to maintain equimolar pooling.

Store any unused, non-pooled sample at 4°C for future use. If one or more samples in a pool have insufficient sequencing yield to generate the desired assembly, stored ligated samples may be re-pooled at a lower plex level or at a higher relative amount.

Proceed directly to Ampure PB Bead Purification of Pooled SMRTbell libraries.



Express Microbial Multiplexing Calculator

Sample Information

Calculate

Plex (Calculated): 10

Total Sample Pool Volume Post Ligation (μl)

100.0

Sample Name	Barcode	Expected Genome Size (bases)	Avg Shear Size (bases)	Optional Sample Conc (ng/μl)	Calculated Volumes (μl)
Microbe Sample 1	BC1001	4600000	10000		15.1
Microbe Sample 2	BC1002	4830000	10000		15.8
Microbe Sample 3	BC1003	3150000	10000		10.3
Microbe Sample 4	BC1004	3500000	10000		11.5
Microbe Sample 5	BC1005	1700000	10000		5.6
Microbe Sample 6	BC1006	2800000	10000		9.2
Microbe Sample 7	BC1007	1900000	10000		6.2
Microbe Sample 8	BC1008	2320000	10000		7.6
Microbe Sample 9	BC1009	2500000	10000		8.2
Microbe Sample 10	BC1010	3200000	10000		10.5

Total Pooled Genome Size (Mb):

30.5

Master Mix Summary (μL)

[DNA PREP MASTER MIX](#)

DNA Prep Buffer	29.13
NAD	4.13
DNA Prep Additive	4.13
DNA Prep Enzyme	4.13
TOTAL	41.50

[DNA DAMAGE REPAIR MASTER MIX](#)

DNA Damage Repair Mix v2	8.38
Enzyme Dilution Buffer	4.13
TOTAL	12.50

[LIGATION MASTER MIX](#)

Ligation Mix	110
Ligation Additive	3.63
Ligation Enhancer (μL)	3.63
TOTAL	117.26

Figure 2. Microbial Multiplexing Calculator with 10-plex example.

AMPure PB Bead Purification of Pooled SMRTbell Libraries

STEP	✓	Purify DNA	Notes								
		Notes for proper handling of AMPure PB beads: <ul style="list-style-type: none">– Bring AMPure PB beads 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.									
1		Add 0.45X volume of AMPure PB beads to the pooled sample.									
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. 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 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. <ul style="list-style-type: none">– Do not remove the tube from the magnetic rack.– Use sufficient volumes of 80% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL 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. <ul style="list-style-type: none">– 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 the appropriate Elution Buffer volume to the beads to elute the DNA: <table><tr><td></td><td>Elution Volume</td></tr><tr><td>Libraries requiring AMPure for size selection to remove <3 kb SMRTbell templates</td><td>50 µL</td></tr><tr><td>Libraries requiring BluePippin size-selection to remove <6 kb SMRTbell templates</td><td>31 µL</td></tr><tr><td>Libraries requiring no size-selection</td><td>11 µL</td></tr></table> <ul style="list-style-type: none">– Gently finger-tap the tube to mix until beads are uniformly re-suspended. It is important to mix well.– Elute the DNA by letting the mix incubate 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.		Elution Volume	Libraries requiring AMPure for size selection to remove <3 kb SMRTbell templates	50 µL	Libraries requiring BluePippin size-selection to remove <6 kb SMRTbell templates	31 µL	Libraries requiring no size-selection	11 µL	
	Elution Volume										
Libraries requiring AMPure for size selection to remove <3 kb SMRTbell templates	50 µL										
Libraries requiring BluePippin size-selection to remove <6 kb SMRTbell templates	31 µL										
Libraries requiring no size-selection	11 µL										
13		For AMPure PB bead size-selection, proceed to “Remove <3 kb SMRTbell Templates using AMPure PB Beads”. This is highly recommended for the Sequel II System. For BluePippin size-selection, proceed to “Optional BluePippin size-selection”. For libraries requiring no size-selection, proceed to “Quantification and Qualification of Final Pooled SMRTbell Libraries”. This is not recommended for the Sequel II System.									

Remove <3 kb SMRTbell Templates using AMPure PB Beads

This AMPure PB bead purification step removes SMRTbell templates <3 kb. AMPure PB beads are diluted to 40% (volume/volume) with Elution Buffer. For effective size-selection, the DNA concentration of SMRTbell libraries to be size selected must be 0.5-10 ng/μL. Using higher concentrations (>15-100 ng/μL) decreases the efficiency of short insert SMRTbell template reduction. Adjust the sample concentration so that the DNA concentration is within this range.

The final AMPure PB bead concentration is critical to the success of this procedure. Therefore, accurate pipetting is of utmost importance to achieve a final 40% AMPure PB bead in Elution Buffer.

Reagent	Volume	✓	Notes
Elution Buffer	3.0 mL		
AMPure PB Bead (stock reagent, thoroughly mixed)	2.0 mL		
Total Volume	5.0 mL		

1. Bring AMPure PB beads stock to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 3.0 mL of the Elution Buffer into a 15 mL conical tube.
4. Add 2.0 mL of the stock AMPure PB beads to the Elution Buffer. When pipetting the viscous AMPure PB bead solution, pipette slowly to ensure that the volume aspirated is as precise as possible. Large residual AMPure solution adhering to the tip should be removed prior to addition to the Elution Buffer.
5. Vortex the diluted AMPure PB bead solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 weeks for future use.

STEP	✓	Purify SMRTbell Templates	Notes
1		Before starting with the procedure, sample concentration must be between 0.5 – 10 ng/μL. The most optimal concentration is 10 ng/μL. Dilute the sample if necessary.	
2		Measure the total sample volume accurately with a pipettor.	
3		To remove <3 kb SMRTbell templates, use 2.2X of diluted AMPure PB beads to the sample.	
4		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. For larger volumes, use a bigger tip. It is important to mix well.	
5		Quickly spin down the tube (for 1 second) to collect the beads.	
6		Incubate the mix on bench top for 15 minutes at room temperature.	
7		Spin down the tube (for 1 second) to collect beads.	
8		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
9		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the beads.	
10		<p>Wash beads with freshly prepared 80% ethanol.</p> <p>Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results.</p> <ul style="list-style-type: none"> – Do not remove the tube from the magnetic rack. – 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). 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. 	
11		Repeat step 10 .	
12		<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> – 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. Beads will be on the side of the tube. – Pipette off any remaining 80% ethanol. 	
13		Check for any remaining droplets in the tube. If droplets are present, repeat step 12 .	
14		<p>Add 11 μL Elution Buffer volume to the beads. When adding 10 μL EB, dispense the volume directly to the beads. Do not let the beads dry. Pipette mix 15 times with wide-bore pipette tips.</p> <ul style="list-style-type: none"> – 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. 	
15		<p>Proceed to the Quantification and Qualification of Final Pooled SMRTbell Libraries step below.</p> <p>Purified SMRTbell libraries may be stored at -20°C.</p>	

Optional BluePippin Size-selection

If desired, size-select your pooled SMRTbell library using the BluePippin system. For BluePippin size-selection, the average pooled library insert size should be approximately 12.5-15 kb. Size-selection may improve assembly of microbial genomes with long repeat regions of >6 kb. BluePippin size-selection cut-off recommendations (BPstart values) are provided in Table 4 below, based on the quantity of the pooled SMRTbell library.

Pooled SMRTbell Library Amount (µg)	BPstart (bases)
>2	7000
≤2	6000

Table 4. Size-selection recommendations based on pooled library characteristics.

Quantify Samples for Size-Selection

1. Dilute 1 µL from the 31 µL Ampure PB bead purified, pooled SMRTbell library from Step 13 of the AMPure PB Bead Purification of Pooled SMRTbell Libraries section into 9 µL of 1X Elution Buffer.
2. Use 1 µL of this 1:10 dilution to determine DNA concentration using a Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer.

Optional: Use the remaining 9 µL of the 1:10 dilution to evaluate the size distribution of the sheared gDNA by standard gel or field inversion agarose or capillary gel electrophoresis. In addition to the methods described earlier, the Agilent Bioanalyzer may also be used to check the size distribution of pooled samples at this step.

Size-Selection

1. Add **10 µL** of Loading Solution to each 30 µL sample remaining from the previous step above ("Quantify Samples for Size-Selection") and mix well by gentle pipetting.
2. Perform size-selection using Sage Sciences' BluePippin system according to the manufacturer's recommendations. Use the *0.75% DF Marker S1 High-Pass 6 kb – 10 kb v3* run protocol and S1 marker. Enter the BPstart values indicated in Table 4 above based on the size-distribution and quantity of your pooled sample.

Note: Visit Sage's website (<http://www.sagescience.com>) to verify that your BluePippin software is up-to-date.

Collect Eluate

1. Wait at least 30 minutes after the run has completed to collect your eluate. Collect the eluate into a 1.5 mL LoBind tube.
2. After collecting the eluate, wash the eluate chamber with 40 µL of Sage Science's 0.1% Tween-20 Wash Solution and then combine the recovered wash solution with the eluted sample. Washing the elution well may further increase recovery yields by approximately 10-20%.
3. Measure the volume of your size-selected sample (eluate plus wash). Adjust volume of eluted sample to 100 µL with 1X Elution Buffer before proceeding to the Final AMPure PB Bead Purification of BluePippin Size-Selected Library step below.

Final AMPure PB Bead Purification of BluePippin Size-Selected Library

STEP	✓	Purify DNA	Notes
		<p>Notes for proper handling of AMPure PB beads:</p> <ul style="list-style-type: none"> – Bring AMPure PB beads to room temperature before use. – AMPure PB bead purification steps should be performed at room temperature. – Before using, mix the bead reagent well until the solution appears homogeneous. – Pipette the reagent slowly since the bead mixture is viscous and precise 	
1		Add 0.45X volume of AMPure PB beads to the pooled sample.	
2		Mix the bead/DNA solution thoroughly by pipette mixing 15 times with wide-bore pipette tips. 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 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		<p>Wash beads with freshly prepared 80% ethanol.</p> <p>Note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results.</p> <ul style="list-style-type: none"> – Do not remove the tube from the magnetic rack. – 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). 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		<p>Remove residual 80% ethanol.</p> <ul style="list-style-type: none"> – 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		<p>Elute purified DNA from the beads:</p> <ul style="list-style-type: none"> – Immediately add the 11 µL Elution Buffer volume to your beads. Pipette mix 15 times with wide-bore pipette tips. It is important to mix well. – Elute the DNA by letting the mix incubate 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. 	
13		<p>Proceed to the Quantification and Qualification of Final Pooled SMRTbell Libraries step below.</p> <p>Purified SMRTbell libraries may be stored at -20°C.</p>	

Quantification and Qualification of Final Pooled SMRTbell Libraries

1. Dilute 1 μL of the final pooled SMRTbell library into 9 μL of 1X Elution Buffer. Use 1 μL of this 1:10 dilution to determine DNA concentration using a Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer. Expected overall yields of non-size-selected pooled libraries are approximately 20%. For size-selected libraries, expect overall yields of approximately 10% depending on the size distribution of the sheared gDNAs.
2. Use the remaining 9 μL of 1:10 dilution to evaluate the size distribution of the final pooled SMRTbell libraries by standard gel or field inversion agarose or capillary gel electrophoresis. In addition to the methods described earlier, the Agilent Bioanalyzer may also be used to check the size distribution of pooled samples at this step.
3. Enter the empirically determined average library insert size in SMRT Link Sample Setup to prepare samples for the annealing and binding steps (see below).

Anneal and Bind SMRTbell Library

Sequel System:

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 located [here](#).

Sequel II System:

Sample setup requirements for microbial multiplexing on the Sequel II System are summarized below:

Primer to use	Sequencing Primer V4
Primer:Template Ratio to use	20:1
Binding Kit to use	Sequel II Binding Kit 2.0
Polymerase:Template Ratio to use	10:1

To enable preparation of binding reactions with a 10:1 polymerase-to-template ratio, enter the following parameter information below in SMRT Link Sample Setup:

Sample Setup Parameter	Parameter Value to Select	Purpose
Sequencing Primer	"Sequencing Primer V2"	Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup
Sequencing Mode	"CCS Reads"	Enables calculation of 10:1 polymerase to template ratio in SMRT Link Sample Setup

After entering all the required parameter information, follow the instructions in SMRT Link Sample Setup to anneal the microbial multiplexing samples with Sequencing Primer V4 and to bind the samples with Sequel II Binding Kit 2.0.

Appendix 1: Master Mix Tables for Setting Up 2-3 Plex Microbial Samples for Sequencing on the Sequel System

Not Recommended for the Sequel II System.

For preparing 2- and 3-plex microbial samples for sequencing on the Sequel System, we recommend starting with twice the amount of input gDNA (i.e., use 2.0 ug of input gDNA to ensure sufficient quantity of final pooled SMRTbell library for sequencing). Throughout the procedure, use double the standard volumes of input DNA and master mix reagents at each step for 2- and 3-plexes, as indicated below. This procedure is not recommended for the Sequel II System.

DNA Prep Master Mix	Vol. (μL)	
Plex Level	2	3
DNA Prep Buffer (μL)	11.7	17.5
NAD (μL)	1.7	2.5
DNA Prep Additive (Pre-diluted 1:5 in Enzyme Dilution Buffer) (μL)	1.7	2.5
DNA Prep Enzyme	1.7	2.5
DNA Damage Repair Master Mix	Vol. (μL)	
Plex Level	2	3
DNA Damage Repair Mix v2 (μL)	3.4	5.0
Enzyme Dilution Buffer (μL)	1.7	2.5
Ligation Master Mix	Vol. (μL)	
Plex Level	2	3
Ligation Mix (μL)	44	66
Ligation Additive (μL)	1.5	2.2
Ligation Enhancer (μL)	1.5	2.2

Appendix 2: Barcoded Overhang Adapters for Sequel II

The following Barcoded Overhang Adapters are recommended for multiplexing in the Sequel II System. The sequences are available [here](#) and may be ordered from any Oligo Synthesis company.

	Barcode ID		Barcode ID		Barcode ID		Barcode ID
1	bcAd1007T	13	bcAd1040T	25	bcAd1059T	37	bcAd1077T
2	bcAd1023T	14	bcAd1043T	26	bcAd1060T	38	bcAd1078T
3	bcAd1024T	15	bcAd1044T	27	bcAd1061T	39	bcAd1079T
4	bcAd1026T	16	bcAd1046T	28	bcAd1062T	40	bcAd1081T
5	bcAd1027T	17	bcAd1047T	29	bcAd1064T	41	bcAd1082T
6	bcAd1028T	18	bcAd1050T	30	bcAd1065T	42	bcAd1083T
7	bcAd1029T	19	bcAd1052T	31	bcAd1066T	43	bcAd1084T
8	bcAd1031T	20	bcAd1053T	32	bcAd1067T	44	bcAd1087T
9	bcAd1033T	21	bcAd1054T	33	bcAd1068T	45	bcAd1091T
10	bcAd1034T	22	bcAd1055T	34	bcAd1070T	46	bcAd1092T
11	bcAd1036T	23	bcAd1057T	35	bcAd1074T	47	bcAd1094T
12	bcAd1037T	24	bcAd1058T	36	bcAd1076T	48	bcAd1095T

Revision History (Description)	Version	Date
Initial release.	01	February 2019
Updated link to Calculator and QRC.	02	February 2019
Updated Procedure to include multiplex recommendations for the Sequel II System.	03	November 2019
Clarified references to various steps within the document.	04	November 2019
Added annealing procedure for barcoded overhang adapters.	05	December 2019

For Research Use Only. Not for use in diagnostic procedures. © Copyright 2019, Pacific Biosciences of California, Inc. All rights reserved. Information in this document is subject to change without notice. Pacific Biosciences assumes no responsibility for any errors or omissions in this document. Certain notices, terms, conditions and/or use restrictions may pertain to your use of Pacific Biosciences products and/or third party products. Please refer to the applicable Pacific Biosciences Terms and Conditions of Sale and to the applicable license terms at <https://www.pacb.com/legal-and-trademarks/terms-and-conditions-of-sale/>. Pacific Biosciences, the Pacific Biosciences logo, PacBio, SMRT, SMRTbell, Iso-Seq and Sequel are trademarks of Pacific Biosciences. BluePippin and SageELF are trademarks of Sage Science, Inc. NGS-go and NGSengine are trademarks of GenDx. FEMTO Pulse and Fragment Analyzer are trademarks of Advanced Analytical Technologies. All other trademarks are the sole property of their respective owners.