Procedure & Checklist – Preparing Multiplexed Microbial SMRTbell® Libraries for the PacBio® Sequel® System

Before You Begin

This procedure is for preparing multiplexed SMRTbell libraries for sequencing on the Sequel System. For each microbe, 1.5 micrograms of gDNA of sufficient quality is required. For optimal results, input gDNA must migrate predominantly above 20 kb as determined by pulsed-field or field inversion gel or capillary electrophoresis. To ensure even coverage across the genome, be sure to isolate gDNA from bacterial cultures which have reached stationary growth phase. Plasmids may occasionally be sequenced and assembled along with the chromosomal DNA. However, plasmids often need to be isolated separately as these may be excluded depending on the extraction kit used, particularly since supercoiled plasmids are also resistant to shearing.

This procedure includes instructions for multiplexing a minimum of two to a maximum of sixteen genomes, up to a total sum of genome sizes of 30 MB. Options for preparing size-selected libraries are also provided. Size-selection may improve assembly of microbial genomes with long repeat regions of >6 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.

- For 2- and 3-plexes **only**, we recommend starting with twice the input DNA amount recommended above (i.e. 3 micrograms of input gDNA to ensure sufficient quantity of final pooled SMRTbell library for sequencing). Throughout the procedure, you will use double the standard volumes of input DNA and master mix reagents at each step for 2- and 3-plexes, as indicated in the tables and instructions below.
- This protocol requires accurate pipetting of small volumes. For pipetting ≤2 µL, use a P2 that has been recently calibrated and is in good working order.

Materials Needed

Item	Usage	Vendor	Part Number
AMPure PB Beads	Sample Purification	PacBio	100-265-900
Template Prep Kit 1.0-SPv3	SMRTbell Library Construction	PacBio	100-991-900
Barcoded Adapter Kit 8A and/or Barcoded Adapter Kit 8B	SMRTbell Library Construction	PacBio PacBio	101-081-300 101-081-400
gTUBE	SMRTbell Library Construction	Covaris	520079
Qubit™Fluorometer	Quantification	ThermoFisher Scientific	Q33226
Qubit™ dsDNA HS Assay Kit	Quantification	ThermoFisher Scientific	Q32854
BluePippin with dye-free, 0.75% Agarose Cassettes and S1 Marker (Optional)	BluePippin size selection	Sage Science	BLF7510
DNA Lo Bind microfuge tubes	SMRTbell Library Construction	Eppendorf	022431021
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA and/or Pippin Pulse Electrophoresis Power Supply and/or FEMTO *Pulse*® Automated Pulsed-Field CE Instrument 2100 Bioanalyzer Instrument	Qualification	Bio-Rad Sage Science Advanced Analytical Technologies Agilent	170-3670 PPI0200 FPv1-CE2 G2939BA

Barcoded Adapters

To perform this procedure, use barcoded adapters provided in the PacBio Barcoded Adapter Kit 8A or 8B. These adapters were chosen based on maximum sequence separation, oligonucleotide purity, and ligation efficiency. The FASTA file can be directly downloaded from our Multiplexing webpage (Sequel_RSII_16_Barcodes_v2.fasta), instructions for importing into SMRT Link if not already included in the menu of options is detailed in the Analysis Procedure – Multiplexed Microbial Assembly with SMRT Link v5.1.0.

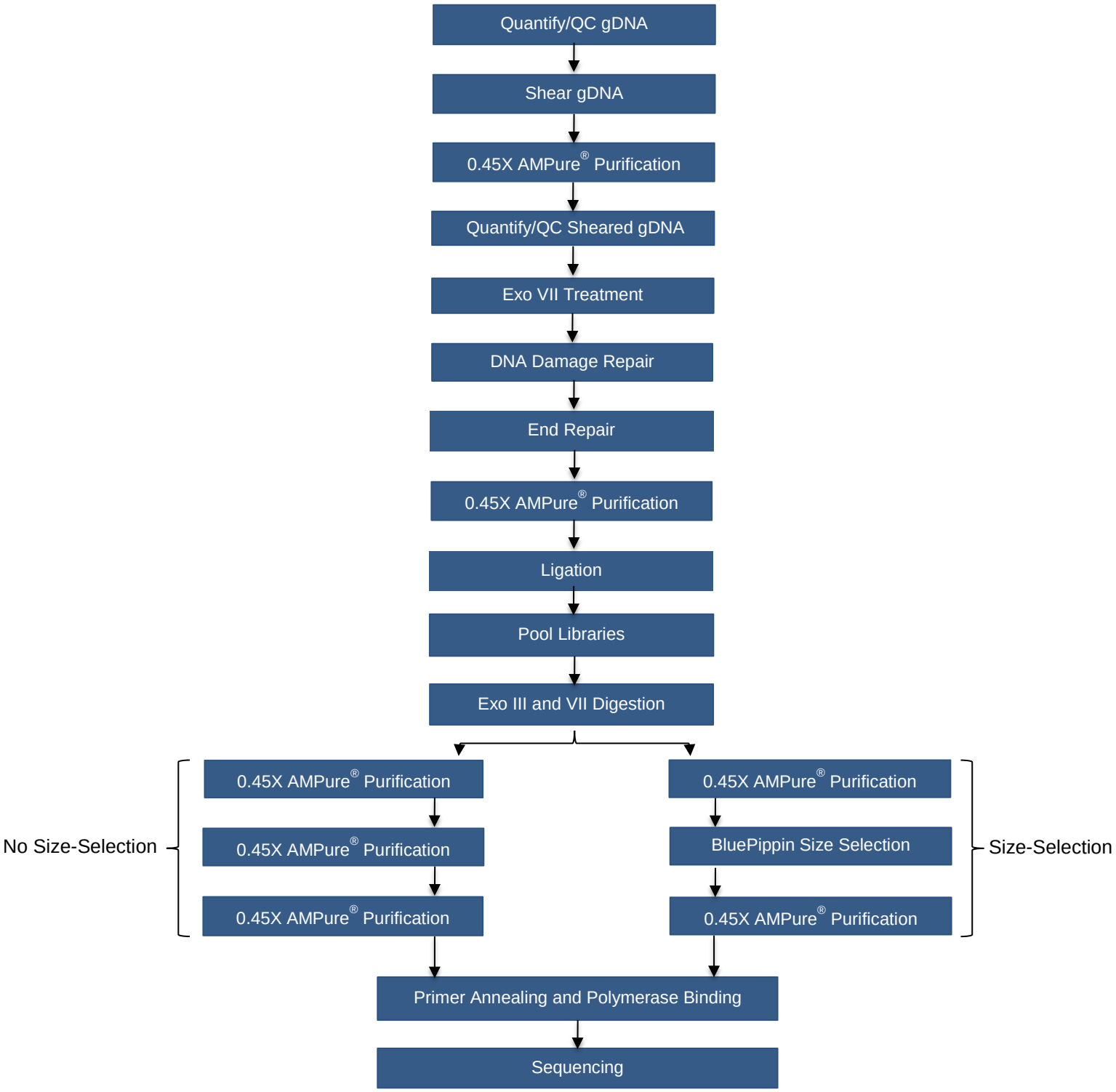
Barcoded Adapter Kit 8A	Barcoded Adapter Kit 8B
bcAd1001	bcAd1015
bcAd1002	bcAd1016
bcAd1004	bcAd1017
bcAd1008	bcAd1018
bcAd1009	bcAd1019
bcAd1010	bcAd1020
bcAd1012	bcAd1021
bcAd1014	bcAd1022

Table 1: Configuration of Barcoded Adapter Kits 8A and 8B

Workflow

In this procedure, individual samples are sheared, quantified and Exo VII-treated before independently going through DNA Damage Repair and End-Repair. After End-Repair, barcoded adapters are ligated to each sample separately. Following ligation, samples are pooled, then digested with Exo III and Exo VII. Size selection may be performed after samples are pooled.

The workflow below summarizes this procedure.



gDNA Quantification and Qualification

Measure gDNA concentration using a Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer. To assess gDNA quality, we recommend pulsed field or field inversion gel or capillary electrophoresis. The Bio-Rad® CHEF Mapper® XA Pulsed Field Electrophoresis procedure can be found here. Recommendations for using Sage Science's Pippin Pulse Electrophoresis Power Supply are available here. The Advanced Analytical Technologies, Inc. FEMTO Pulse automated pulsed-field capillary electrophoresis instrument recommendations are also available here.

Suitable gDNA should migrate predominantly above 20 kb.

Shear Genomic DNA Using a Covaris g-TUBE

STEP	✓	Shear DNA	Notes
1		Dilute **1.5 µg** gDNA into **150 µL** 1X Elution Buffer to a final concentration of 10 ng/µL. For 2- and 3-plexes **only**: Dilute **3 µg** gDNA into **300 µL** 1X Elution Buffer. **Note:** Depending upon the quality of your sample, approximately 20-50% sample loss is to be expected as a result of the shearing and concentration process. Therefore, be sure to have sufficient amounts of starting DNA to achieve 75 ng/µL of concentrated and sheared DNA for the Exo VII treatment step.	
2		Transfer gDNA to the g-TUBE and centrifuge at 2400 x g (6000 rpm in the Eppendorf MiniSpin Plus) for 2 minutes.	
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 2400 x g (6000 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.	

AMPure PB Bead Purification of Sheared gDNA

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **68 µL** of AMPure PB magnetic beads (**0.45X**) to each **150 µL** sheared gDNA sample. For 2- and 3-plexes **only**: Add **135 µL** AMPure PB magnetic beads (**0.45X**) to each **300 µL** sheared gDNA sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes at room temperature.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional**: Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	

5		Elute purified DNA from the beads: ● Add **15 µL** 1X Elution Buffer to the tube, finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. For 2- and 3-plexes **only**: Add **30 µL** 1X Elution Buffer to the tube, finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer the supernatant containing purified DNA to a fresh 1.5 mL DNA Lo-Bind microfuge tube. ● Discard the beads.	

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 or field inversion agarose or capillary gel electrophoresis. The average size of your sheared gDNA should be approximately 10 kb. If DNA is over-sheared, decrease centrifugation speed. If DNA is under-sheared, increase centrifugation speed.

Note: If the concentration of sheared and AMPure PB purified gDNA is <75 ng/µL 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 ≥75 ng/µL.
- If additional gDNA is not available and concentrations of all samples after shearing and AMPure PB bead purification is ≥37.5 ng/µL, 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 less than ~2000 ng for non-size-selected libraries or ~4000 ng for size-selected libraries. For example, starting with 37.5 ng/µL sheared gDNA for each microbe in an 8-plex would give a total input DNA mass of 3960 ng (13.2 µL * 37.5 ng/µL * 8).
- If additional gDNA is not available and the concentration of one or more samples after shearing and AMPure PB bead purification is <37.5 ng/µL, 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 perform the optional sample quantitation step here. Enter the empirically-determined sample concentrations into the Microbial Multiplexing Calculator. Note that proceeding with this option may increase variation in relative coverage among the microbes in the pool.

Exo VII Treatment of DNA

If necessary, adjust sheared gDNA concentration to 75 ng/µL with 1X Elution Buffer, then aliquot 13.2 µL of each sheared gDNA sample at 75 ng/µL into a fresh Lo-Bind tube.

For 2- and 3-plexes **only**: aliquot 26.4 µL of each sheared gDNA sample at 75 ng/µL into a fresh Lo-Bind tube.

Prepare Exo VII Master Mix according to the table below or the Master Mix Tables provided in the Microbial Multiplexing Calculator. Note that indicated volumes include 25% overage, and for 2- and 3-plexes only, have been doubled. For reference, reagent volumes for all Master Mixes and Plex Levels are also provided in Appendix I.

Reagent	Tube Cap Color	2 or 4 Plex	3 or 6 Plex	8 Plex	12 Plex	16 Plex	N Plex
DNA Damage Repair Buffer		7.5	11.3	15.0	22.5	30.0	N x 1.5 x 1.25
Exo VII		1.5	2.3	3.0	4.5	6.0	N x 0.3 x 1.25

1. Add 1.8 µL Exo VII Master Mix to each 13.2 µL individual sheared gDNA sample, for a total volume of 15 µL.

   For 2- and 3-plexes **only**: Add 3.6 µL Exo VII Master Mix to each 26.4 µL individual sheared gDNA sample, for a total volume of 30 µL.
2. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
3. Incubate at 37ºC for 15 minutes.

Repair DNA Damage

1. Prepare DNA Damage Master Mix according to the table below or the Master Mix Tables provided in the Microbial Multiplexing Calculator. Note that indicated volumes include 25% overage, and for 2- and 3-plexes only, have been doubled. For reference, reagent volumes for all Master Mixes and Plex Levels are also provided in Appendix I.

Reagent	Tube Cap Color	2 or 4 Plex	3 or 6 Plex	8 Plex	12 Plex	16 Plex	N Plex
1X Elution Buffer (µL)		6.5	9.8	13.0	19.5	26.0	N x 1.3 x 1.25
DNA Damage Repair Buffer (µL)		2.5	3.8	5.0	7.5	10.0	N x 0.5 x 1.25
ATP high (µL)		10.0	15.0	20.0	30.0	40.0	N x 2.0 x 1.25
dNTPs (µL)		1.0	1.5	2.0	3.0	4.0	N x 0.2 x 1.25
NAD (µL)		1.0	1.5	2.0	3.0	4.0	N x 0.2 x 1.25
DNA Damage Repair Mix (µL)		4.0	6.0	8.0	12.0	16.0	N x 0.8 x 1.25

2. Add 5 µL DNA Damage Master Mix to each individual 15 µL of Exo VII-treated sample, for a total volume of 20 µL.

   For 2- and 3-plexes **only**: Add 10 µL DNA Damage Master Mix to each individual 30 µL of Exo VII-treated sample, for a total volume of 40 µL.
3. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
4. Incubate at 37℃ for 60 minutes .

End-Repair

1. Add 1 µL End Repair Mix to each individual 20 µL sample, for a total volume of 21 µL.

   For 2- and 3-plexes **only**: Add 2 µL End Repair Mix to each individual 40 µL sample, for a total volume of 42 µL.
2. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
3. Incubate at 20℃ for 5 -minutes.

4. Add **79 µL** of 1X Elution Buffer to each **21 µL** End-Repaired sample for a volume of **100 µL**.
   For 2- and 3-plexes **only**: Add **58 µL** 1X Elution Buffer to each **42 µL** End-Repaired sample for a volume of **100 µL**.
5. Proceed to AMPure PB Bead Purification.

AMPure PB Bead Purification

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **45 µL** of AMPure PB magnetic beads (**0.45X**) to each **100 µL** sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional***:* Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	

5		Elute purified DNA from the beads: ● Add **12 µL** 1X Elution Buffer (or **13 µL** 1X Elution Buffer if including optional quantification of sample at this stage*) to the tube. For 2- and 3-plexes **only**: Add **23 µL** 1X Elution Buffer (or **24 µL** 1X Elution Buffer if including optional quantification of sample at this stage*) to the tube. **If unequal amounts of sheared DNA were input into Exo VII digestion, you **must** quantify your samples at this stage.* ● Finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer **11.2 µL** (or **12.2 µL** if including optional quantification of sample at this stage) of the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. For 2- and 3-plexes **only**: Transfer **22.4 µL** (or **23.4 µL** if including optional quantification of sample at this stage) of the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. ● Discard the beads.	

Optional: Quantify and QC Samples Post End-Repair

Important: If unequal amounts of sheared DNA were input into Exo VII digestion, you **must** quantify your samples at this stage:

- 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 Qubit fluorometer and Qubit HS DNA reagents as recommended by the manufacturer.
- Enter the empirically-determined sample concentrations into the Microbial Multiplexing Calculator before pooling samples.

Ligate Blunt Barcoded Adapters

1. To each 11.2 µL sample, add 2 µL of 20 µM Barcoded Blunt Adapter for a total volume of 13.2 µL.

   For 2- and 3-plexes **only**: To each 22.4 µL sample, add 4 µL 20 µM Barcoded Blunt Adapter for a total volume of 26.4 µL.
2. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
3. Prepare Ligation Master Mix according to the table below or the Master Mix Tables provided in the Microbial Multiplexing Calculator. Note that indicated volumes include 25% overage, and for 2- and 3-plexes only, have been doubled. For reference, reagent volumes for all Master Mixes and Plex Levels are also provided in Appendix I.

Reagent	Tube Cap Color	2 or 4 Plex	3 or 6 Plex	8 Plex	12 Plex	16 Plex	N Plex
Template Prep Buffer		8.0	12.0	16.0	24.0	32.0	N x 1.6 x 1.25
ATP Low		4.0	6.0	8.0	12.0	16.0	N x 0.8 x 1.25
Ligase		2.0	3.0	4.0	6.0	8.0	N x 0.4 x 1.25

4. Add 2.8 µL Ligation Master Mix to each individual 13.2 µL sample, for a total volume of 16 µL.
   For 2- and 3-plexes **only**: Add 5.6 µL Ligation Master Mix to each individual 26.4 µL sample, for a total volume of 32 µL.

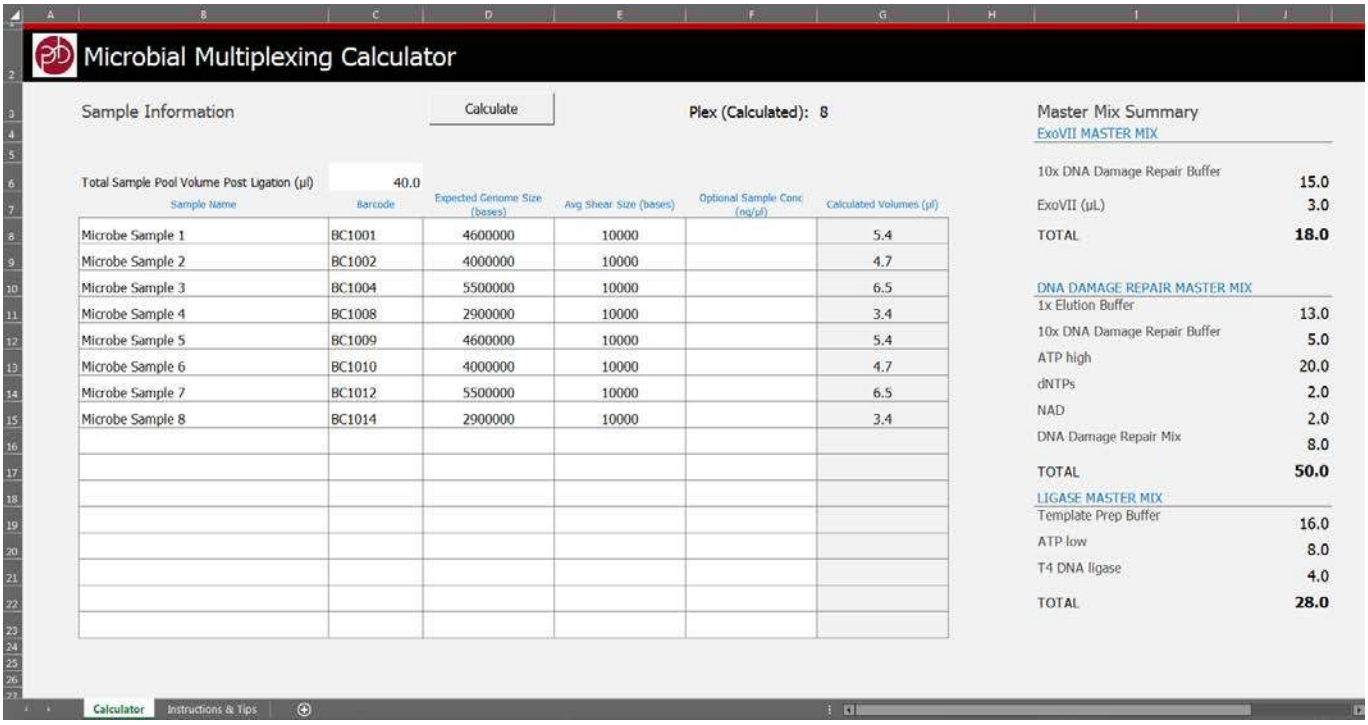
5. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
6. Incubate at 20℃ for 30 minutes. Ligation reactions may be extended overnight if desired.
7. Heat-inactivate the ligase by incubating samples at 65℃ for 10 minutes. Heat -inactivate the ligase **before** proceeding to pooling samples (next step).

Sample Pooling

Pool samples for Exo III/Exo VII digestion according to the volumes provided by the Microbial Multiplexing Calculator. An example of pooling volumes for a typical 8-plex is shown in Figure 1 below. The recommended pooling volume for non-size selected samples is 40 µL. If you plan to perform size-selection on your sample **or** have input less than 1 µg of sheared DNA for any sample into the initial Exo VII digestion step, increase the recommended pool volumes to 60-80 µL to ensure sufficient final pooled barcoded SMRTbell template yield for sequencing.

If unequal amounts of sheared DNA, or less than 1 µg of sheared DNA for any sample, were input into the initial Exo VII digestion, be sure to also enter the empirically-determined sample concentrations into the Optional Sample Concentration row of the Calculator.

Store any unused, non-pooled sample at -20℃ 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.



Microbial Multiplexing Calculator

Sample Information | Calculate | Plex (Calculated): 8

Total Sample Pool Volume Post Ligation (µl): 40.0

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		5.4
Microbe Sample 2	BC1002	4000000	10000		4.7
Microbe Sample 3	BC1004	5500000	10000		6.5
Microbe Sample 4	BC1008	2900000	10000		3.4
Microbe Sample 5	BC1009	4600000	10000		5.4
Microbe Sample 6	BC1010	4000000	10000		4.7
Microbe Sample 7	BC1012	5500000	10000		6.5
Microbe Sample 8	BC1014	2900000	10000		3.4

Master Mix Summary

ExoVII MASTER MIX

10x DNA Damage Repair Buffer	15.0
ExoVII (µL)	3.0
TOTAL	18.0

DNA DAMAGE REPAIR MASTER MIX

1x Elution Buffer	13.0
10x DNA Damage Repair Buffer	5.0
ATP high	20.0
dNTPs	2.0
NAD	2.0
DNA Damage Repair Mix	8.0
TOTAL	50.0

LIGASE MASTER MIX

Template Prep Buffer	16.0
ATP low	8.0
T4 DNA ligase	4.0
TOTAL	28.0

Calculator | Instructions & Tips

Figure 1. Microbial Multiplexing Calculator with 8-plex example.

Exo III and VII Digestion

This step improves library quality by removing failed ligation products.

To each 40 µL pooled sample from the Pooling step above, add the reagents shown below. If pooled sample volume is greater than 40 µL, scale Exo III and Exo VII volumes proportionally.

Calculate the volumes of Exo III and Exo VII to be used according to this formula: [N ÷ 40 µL * 1µL].

For example, use 1.5 µL of Exo III and 1.5 µL of Exo VII for a 60 µL pooled sample.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Exo III		100.0 U/µL		1.0 µL
Exo VII		10.0 U/µL		1.0 µL
Total Volume				42.0 µL

1. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
2. Incubate at 37℃ for 60 minutes.
3. If necessary, add 1X Elution Buffer to the pooled sample for a minimum volume of **100 µL** before AMPure PB Bead purification. For example, to a 40 µL pooled sample, add 60 µL 1X Elution Buffer.

   Note: You must proceed immediately with purification after exonuclease digestion.

AMPure PB Bead Purification of Pooled SMRTbell Libraries

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **0.45X** volume of AMPure PB beads to the pooled sample. For example, use **45 µL** AMPure PB beads for a **100 µL** pooled sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional**: Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	
5		Elute purified DNA from the beads (Note: For samples for size-selection, elute in 31 µL 1X Elution Buffer. If you do not plan to size-select your sample, elute in 100 µL 1X Elution Buffer.) ● Add **100 µL** 1X Elution Buffer (for no size selection) or **31 µL** 1X Elution Buffer (for size selection) to the tube. ● Finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. ● Discard the beads.	

To proceed with a non-size-selected sample, refer to the next step. To size-select your sample, proceed to the Optional Blue Pippin Size-Selection step.

Second AMPure PB Bead Purification of Pooled SMRTbell Libraries (No Size-Selection)

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **45 µL** volume of AMPure PB beads (**0.45X**) to each **100 µL** sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional**: Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	
5		Elute purified DNA from the beads: ● Add **100 µL** 1X Elution Buffer to the tube. ● Finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. ● Discard the beads.	

Third and Final AMPure PB Bead Purification of Pooled SMRTbell Libraries (No Size-Selection)

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **45 µL** volume of AMPure PB beads (**0.45X**) to each **100 µL** sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional**: Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	
5		Elute purified DNA from the beads: ● Add **10 µL** 1X Elution Buffer to the tube. ● Finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. ● Discard the beads.	

Proceed to Quantification and Qualification of Pooled SMRTbell Libraries.

Optional Blue Pippin Size-selection

If desired, size-select your pooled SMRTbell library. For size-selection, average pooled library insert size should be approximately 10 kb. Size-selection may improve assembly of microbial genomes with long repeat regions of >6 kb. Size-selection cut-off recommendations (BPstart values) are provided in Table 2 below, based on quantity of the pooled library.

Table 2. Size-selection recommendations based on pooled library characteristics.

Pooled Library Amount (µg)	**BPstart (bases)**
>2	7000
<2	6000

Quantify Samples for Size-Selection

1. Dilute 1 µL from the 31 µL Ampure PB bead purified, pooled SMRTbell library from the “Second AMPure PB bead Purification of Pooled SMRTbell Libraries (No Size-Selection)” step into 9 µL 1X Elution Buffer.
2. Use 1 µL of this 1:10 dilution to determine DNA concentration using 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 size distribution of sheared gDNA by standard or field inversion agarose or capillary gel electrophoresis. In addition to the methods described earlier, the Agilent Bioanalyzer may be used to check the size distribution of pooled samples at this step.

Size-Selection

1. Add 10 µL 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' Blue Pippin according to 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 2 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.
2. After collecting eluate, wash eluate chamber with 40 µL 0.1% Tween solution and combine wash with eluate.
3. Measure the volume of your size-selected sample (eluate plus wash). Add 1X Elution Buffer to a final volume of 100 µL before proceeding to AMPure PB bead purification.

Final AMPure PB Bead Purification of Size-Selected Library

STEP	✓	Purification Process	Notes
		Notes for proper handling of AMPure PB beads: ● Bring AMPure PB beads to room temperature before use. ● Vortex for 30 seconds to mix well before each use. ● Always pipette slowly to ensure complete transfer of viscous AMPure PB bead solution.	
1		Bind DNA to beads: ● Add **45 µL** volume of AMPure PB beads (**0.45X**) to each **100 µL** sample. ● Finger tap to mix then very briefly spin to return contents to the bottom of the tube. ● Place on end-over-end rotator for 15 minutes.	
2		Wash beads with 70% ethanol. **Note**: 70% ethanol is hygroscopic and should be prepared **fresh** to achieve optimal results. Store 70% ethanol in a tightly capped polypropylene tube for up to 3 days. ● Very briefly spin to return contents to the bottom of the tube, then place the tube on a magnetic rack and let beads separate fully. ● Remove the supernatant and discard. **Optional**: Instead of discarding, reserve the supernatant in another tube. If DNA is not recovered at the end of this procedure, add an equal volume of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to attempt to recover the DNA. ● Leave the tube on the rack and wash beads by adding 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
3		Repeat 70% ethanol wash. ● Leave the tube on the rack and add 1.5 mL freshly-prepared 70% ethanol. ● Remove ethanol and discard.	
4		After the second wash, remove all traces of ethanol. ● Remove the tube from the rack, spin very briefly, return to the rack and let beads separate. ● Without disturbing the bead pellet, discard any residual ethanol at the bottom of the tube. ● Remove the tube from the rack and air dry the beads for 30 seconds. Do not let the beads dry out.	
5		Elute purified DNA from the beads: ● Add **10 µL** 1X Elution Buffer to the tube. ● Finger tap to resuspend the beads, then spin very briefly to return the contents to the bottom of the tube. ● Let sit at room temperature for 2 minutes. ● Return the tube to the magnetic rack and let the beads separate fully. ● Without disturbing the bead pellet, transfer the supernatant containing purified DNA to a fresh 1.5 mL Lo-Bind microfuge tube. ● Discard the beads.	

Proceed to Quantification and Qualification of Pooled SMRTbell Libraries.

Quantification and Qualification of Pooled SMRTbell Libraries

1. Dilute 1 µL of the final pooled SMRTbell library into 9 µL 1X Elution Buffer. Use 1 µL of this 1:10 dilution to determine DNA concentration using 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 size distribution of sheared gDNAs.
2. Use the remaining 9 uL of 1:10 dilution to evaluate size distribution of final pooled SMRTbell libraries by standard or field inversion agarose or capillary gel electrophoresis.
3. Use empirically determined average library insert size to calculate the molarity for preparation of bound complexes (refer to SMRT Link Sample Set Up for more information).

Anneal and Bind SMRTbell Templates

- For primer annealing, follow the instructions in SMRT Link Sample Setup. Select Sequencing Primer v4.
- For polymerase binding, follow the instructions in SMRT Link Sample Setup. Select Sequel Binding Kit 2.1.

Sequencing

For recommendations for sequencing of microbial multiplex samples, refer to Quick Reference Card – Diffusion Loading and Pre-Extension Time Recommendations for the Sequel System.

Appendix 1: Master Mix Tables

Plex Level	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Plus 25% Overage	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Double for 2- and 3-plex	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1
Multiplier	5	7.5	5	6.25	7.5	8.75	10	11.25	12.5	13.75	15	16.25	17.5	18.75	20

Table 1. Exo VII Master Mix	Vol. (µL)														
Plex Level	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
10x DNA Damage Repair Buffer (µL)	7.5	11.3	7.5	9.4	11.3	13.1	15.0	16.9	18.8	20.6	22.5	24.4	26.3	28.1	30.0
ExoVII (µL)	1.5	2.3	1.5	1.9	2.3	2.6	3.0	3.4	3.8	4.1	4.5	4.9	5.3	5.6	6.0

Table 2. DNA Damage Repair Master Mix	Vol. (µL)														
Plex Level	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1x Elution Buffer	6.5	9.8	6.5	8.1	9.8	11.4	13.0	14.6	16.3	17.9	19.5	21.1	22.8	24.4	26.0
10x DNA Damage Repair Buffer (µL)	2.5	3.8	2.5	3.1	3.8	4.4	5.0	5.6	6.3	6.9	7.5	8.1	8.8	9.4	10.0
ATP high (µL)	10.0	15.0	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.5	30.0	32.5	35.0	37.5	40.0
dNTPs (µL)	1.0	1.5	1.0	1.3	1.5	1.8	2.0	2.3	2.5	2.8	3.0	3.3	3.5	3.8	4.0
NAD (µL)	1.0	1.5	1.0	1.3	1.5	1.8	2.0	2.3	2.5	2.8	3.0	3.3	3.5	3.8	4.0
DNA damage repair mix (µL)	4.0	6.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.0	16.0

Table 3. Ligase Master Mix	Vol. (µL)														
Plex Level	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Template Prep Buffer (µL)	8.0	12.0	8.0	10.0	12.0	14.0	16.0	18.0	20.0	22.0	24.0	26.0	28.0	30.0	32.0
ATP low (µL)	4.0	6.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.0	16.0
T4 DNA ligase (µL)	2.0	3.0	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0

Revision History (Description)	Version	Date
Initial Release (Internal only)	01	April 2018
Initial Full Release	02	May 2018