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 System. For each microbe, 1.0 microgram 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 two to a maximum of sixteen genomes, up to a total sum of genome sizes of 30 MB (including plasmid elements). Options for preparing size-selected libraries are also provided. Size-selection may improve assembly of microbial genomes with long repeat regions of >6 kb. If performing size-selection is desired, the average pooled library insert size should be approximately12.5-15 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.

- For 2- and 3-plexes **only**, we recommend starting with twice the input DNA amount recommended above (i.e 2.0 micrograms 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 in the tables and instructions below.

- This procedure requires accurate pipetting of small volumes. For pipetting ≤2 µL, use a P2 pipetor that has been recently calibrated and is in good working order.

Required Materials

Item	Where Required	Vendor	Part Number
Qualification			
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA and/or	Option 1 for sample high-molecular weight DNA QC	Bio-Rad	170-3670
Pippin Pulse Electrophoresis Power Supply	Option 2 for sample high-molecular weight DNA QC	Sage Science	PP10200
FEMTO *Pulse*® Automated Pulsed Field CE Instrument	Option 3 for sample high-molecular weight DNA QC	Advanced Analytical Technologies	FFv1-CE2
2100 Bioanalyzer Instrument	For measuring sheared gDNA	Agilent	G2939BA
Quantification			
Qubit™ Fluorometer	Highly recommended for accurate quantification	ThermoFisher Scientific	Q33226
Qubit dsDNA HS Assay Kit	Highly recommended for accurate quantification	ThermoFisher Scientific	Q32854
Shearing			
g-TUBE	For shearing	Covaris	10145
SMRTbell Library Construction			
SMRTbell Express Template Prep Kit 2.0	Library Costruction	PacBio	100-938-900
Barcoded Overhang Adapter Kit 8A and/or		PacBio	101-628-400
Barcoded Overhang Adapter Kit 8B		PacBio	101-628-500
DNA Lo Bind microfuge tubes		Eppendorf	22431021
Wide Orifice Tips (Tips LTS W-O 200 UL Fltr RT-L200WFLR)		Rainin	17014294
AMPure PB Beads		PacBio	100-265-900
Size Selection			
BluePippin Size-Selection System	Size selection (optional)	Sage Science	BLU0001
BluePippin with dye free, 0.75% Agarose Cassettes and S1 Marker	Size selection (optional)	Sage Science	BLF7510

Table 1. List of Required Materials and Equipment.

Barcoded Overhang Adapters

To perform this procedure, use barcoded adapters provided in the PacBio Barcoded Overhang Adapter Kit 8A or 8B. To download barcode FASTA sequence files, visit our Analytical Software Multiplexing page.

Barcoded Overhang Adapter Kit -8A	Barcoded Overhang Adapter Kit -8B
bc1001	bc1015
bc1002	bc1016
bc1003	bc1017
bc1008	bc1018
bc1009	bc1019
bc1010	bc1020
bc1011	bc1021
bc1012	bc1022

Table 2. List of Barcoded Overhang Adapters

Workflow

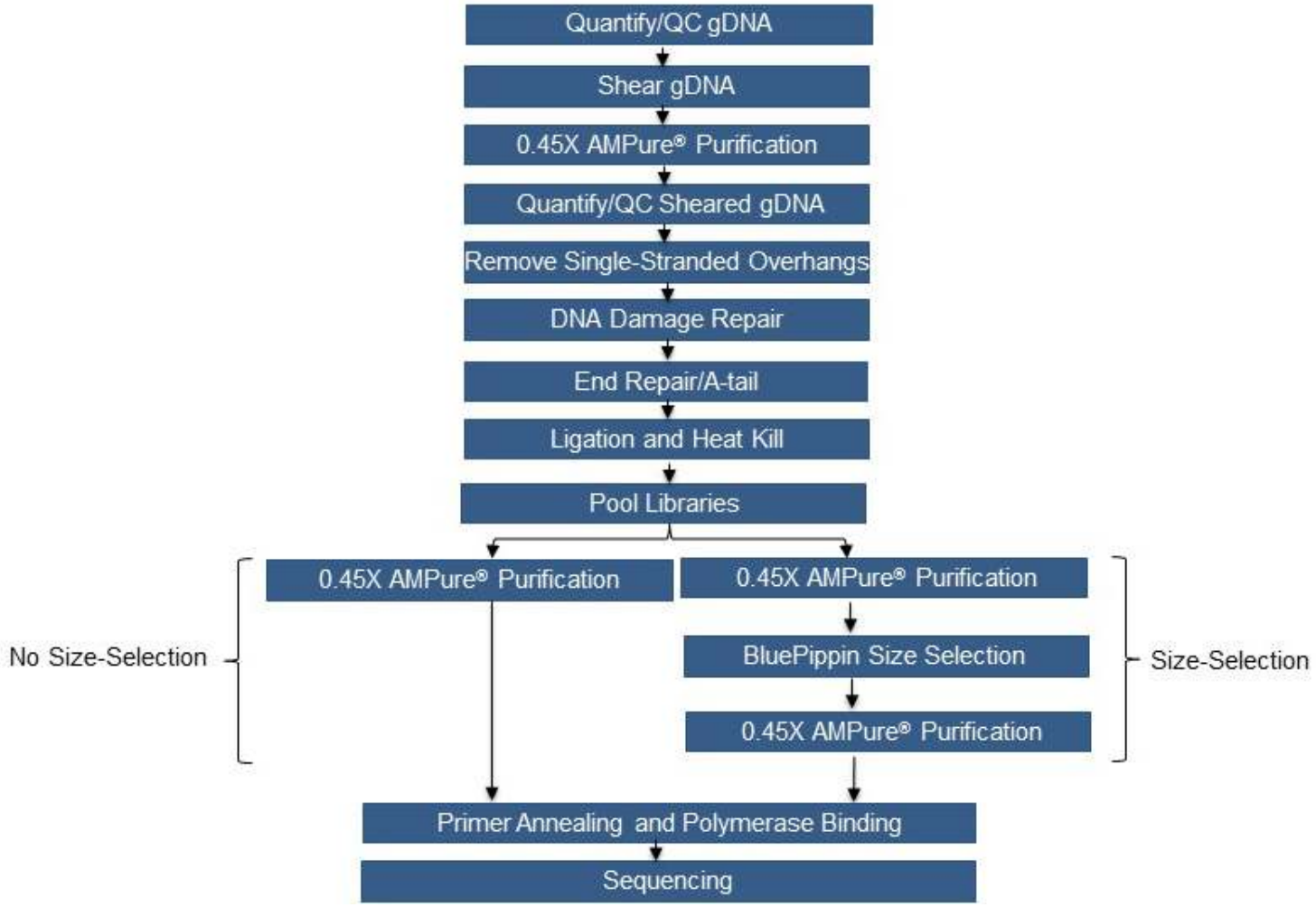


Figure 1: Workflow for preparing multiplexed microbial library using the SMRTbell Express Template Prep Kit 2.0 and Barcoded Overhang Adapter Kit 8A and/or 8B.

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 3 below may be used to evaluate gDNA quality. Links to recommended procedures for each are also provided in the table. Suitable genomic DNA should migrate predominanatly 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
Advanced Analytical Technologies, Inc. FEMTO Pulse	Advanced Analytical Website
Sage Science Pippin Pulse	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

Table 3. gDNA Quality QC Methods and Procedures.

Shear Genomic DNA Using a Covaris g-TUBE

STEP	✓	Shear DNA	Notes
1		Dilute **1.0 µg** gDNA into **100 µL** 1X Elution Buffer to a final concentration of 10 ng/µL. For 2- and 3-plexes **only**: Dilute **2 µg** gDNA into **200 µL** 1X Elution Buffer.	
2		Transfer gDNA to the g-TUBE and centrifuge at 2029 x g (5500 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 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.	

Concentrate sheared gDNA using AMPure PB Beads

STEP	✓	Concentrate DNA	Notes
		Notes for proper handling of AMPure PB beads: – Bring beads AMPure PB bead 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 volumes are critical to the purification process.	
1		Add **45 µL** of AMPure PB Magnetic beads (**0.45X**) to each **100 µL** sheared gDNA sample. – For 2- and 3-plexes **only**: Add **90 µL** AMPure PB magnetic beads (**0.45X**) to each **200 µL** 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		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 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. – 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		Elute purified DNA from the beads (the volume to go into “Remove Single Stranded Overhangs”). – Add **16 µL** of Elution Buffer to your beads. – For 2- and 3-plexes **only**: Add **32 µL** Elution Buffer to your beads.	

13		Add the 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 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.	
14		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 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 <33 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 ≥33 ng/µL.
   - 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 less than ~1000 ng for non-size-selected libraries or ~2000 ng for size-selected libraries. For example, starting with 16.5 ng/µL sheared gDNA for each microbe in an 8-plex would give a total input DNA mass of 1927 ng (14.6 µL * 16.5 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 <16.5 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.

Reagent Handling

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

- Never leaving tubes at room temperature.
- Working on ice at all times when preparing master mixes.
- Finger tapping 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 4: Temperature sensitive reagents

Remove Single-Strand Overhangs

If necessary, 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 fresh Lo-Bind tube.

For 2- and 3-plexes only: aliquot 29.2 µL of each sheared gDNA sample at 33 ng/µL into a fresh Lo-Bind tube.

Prepare the following:

1. The stock DNA Prep Additive must first be diluted. Dilute the stock DNA Prep Additive 1:5 in Enzyme Dilution Buffer (found in the kit). Dilute 1 µL of stock DNA Prep Additive into 4 µL Enzyme Dilution Buffer. The diluted DNA Prep Additive should be used immediately and should not be stored.
2. Prepare DNA Prep Master Mix according to the table below (or the Master Mix Tables provided in the Microbial Multiplexing Calculator). 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 Prep Buffer		11.7	17.5	23.3	35.0	46.6	N X 2.33 X 1.25
NAD		1.7	2.5	3.3	5.0	6.6	N X 0.33 X 1.25
Diluted DNA Prep Additive (step 1 above)		1.7	2.5	3.3	5.0	6.6	N X 0.33 X 1.25
DNA Prep Enzyme		1.7	2.5	3.3	5.0	6.6	N X 0.33 X 1.25

Note that indicated volumes include 25% overage, and for 2- and 3-plexes only, they have been doubled.

3. Add **3.3 µL** DNA Prep Master Mix to each 14.6 µL individual sheared gDNA sample, for a total volume of 17.9 µL.

   For 2- and 3-plexes **only**: Add **6.6 µL** Master Mix to each 29.2 µL individual sheared gDNA sample, for a total volume of 35.8 µL.
4. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
5. Incubate at 37ºC 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).. 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 Mix v2		3.4	5.0	6.7	10.1	13.4	N X 0.67 X 1.25
Enzyme Dilution Buffer		1.7	2.5	3.3	5.0	6.6	N X 0.33 X 1.25

Note that indicated volumes include 25% overage, and for 2- and 3-plexes only, they have been doubled.

2. Add **1 µL** DNA Damage Master Mix to each individual 17.9 µL of sample, for a total volume of 18.9 µL. For 2- and 3-plexes **only**: Add **2 µL** DNA Damage Master Mix to each individual 35.8 µL of sample, for a total volume of 37.8 µL.
3. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
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. Add **1 µL** End Prep Mix to each individual 18.9 µL sample, for a total volume of 19.9 µL.
   For 2- and 3-plexes **only**: Add **2 µL** End Repair Mix to each individual 37.8 µL sample, for a total volume of 39.8 µL.
2. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
3. Incubate at 20°C for 10 minutes, then 65°C for 30 minutes, then return the sample to 4°C. Proceed to the next step.

Ligate Overhang Barcoded Adapters

1. To each 19.9 µL sample, add **2.0 µL** of 17 µM Barcoded Overhang Adapter for a total volume of 21.9 µL. For 2- and 3-plexes **only**: To each 39.8 µL sample, add **4.0 µL** 17 µM Barcoded Overhang Adapter for a total volume of 43.8 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). 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
Ligation Mix		44.0	66.0	88.0	132.0	176.0	N X 10.0 X 1.10
Ligation Additive		1.5	2.2	2.9	4.4	5.8	N X 0.33 X 1.10
Ligation Enhancer		1.5	2.2	2.9	4.4	5.8	N X 0.33 X 1.10

Note that indicated volumes include 10% overage, and for 2- and 3-plexes only, they have been doubled.

4. Add **10.7** µL Ligation Master Mix to each individual 21.9 µL sample, for a total volume of 32.6 µL. For 2- and 3-plexes **only**: Add **21.4** µL Ligation Master Mix to each individual 43.8 µL sample, for a total volume of 65.2 µL.
5. Finger tap to mix then very briefly spin to return contents to the bottom of the tube.
6. Incubate at 20°C for 60 minutes.
7. IMPORTANT: Heat kill the ligase before proceeding to the sample pooling step. To heat kill the ligase, incubate the samples at 65°C for 10 minutes.
8. Proceed to the next step.

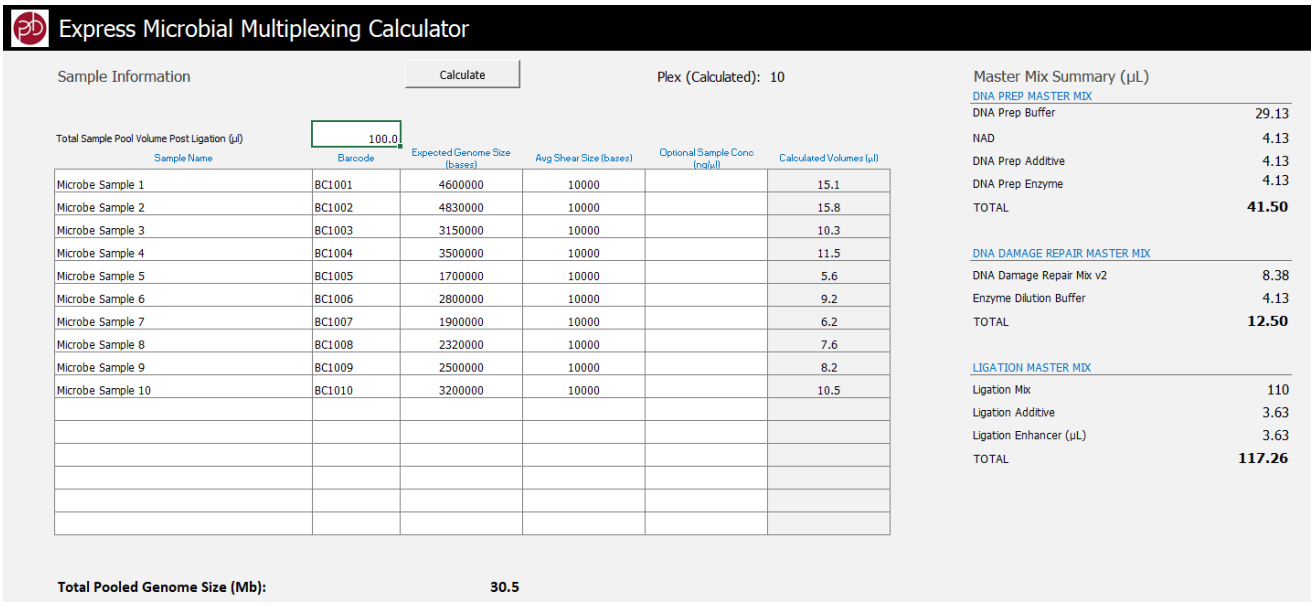
Sample Pooling

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 recommended pooling volume is 100 µL.

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 starting sample concentrations into the Optional Sample Concentration row of the Pooling Calculator to maintain equimolar pooling.

Store any unused, non-pooled sample at -20°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: – Bring beads AMPure PB bead 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 homogenous. – Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
1		Add **45 µL** of AMPure PB Magnetic beads (**0.45X**) to each **100 µL** 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. – 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		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 **the appropriate volume** (see below) of Elution Buffer volume to the beads to elute the DNA. **Elution Volume** **Libraries requiring no size-selection** 11 µL **Libraries requiring size-selection** 31 µL – 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.	

For a non-size-selected sample, proceed to Quantification and Qualification of Pooled SMRTbell Libraries.
To size-select your sample, proceed to the Optional BluePippin Size-Selection step.

Optional BluePippin Size-selection

If desired, size-select your pooled SMRTbell library. For size-selection, 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. Size-selection cut-off recommendations (BPstart values) are provided in Table 4 below, based on quantity of the pooled library.

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

Table 5. 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 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 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** 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 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 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 AMPure PB bead purification.

Final AMPure PB Bead Purification of Size-Selected Library

STEP	✓	Purify DNA	Notes
		Notes for proper handling of AMPure PB beads: – Bring beads AMPure PB bead 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 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. – 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		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		Elute purified DNA from the beads: – Immediately add the **10 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.	

Proceed to Quantification and Qualification of Pooled SMRTbell Libraries below.

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 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. Use the empirically determined average library insert size to calculate the molarity for preparation of annealed and bound complexes (refer to SMRT Link Sample Set Up for more information).

Anneal and Bind SMRTbellTemplates

- 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 the appropriate version of Sequel Binding Kit.

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 here.

Appendix 1: Master Mix Tables

Table 1. DNA Prep Master Mix	Vol. (µL)														
Plex Level	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
DNA Prep Buffer (µL)	11.7	17.5	11.7	14.6	17.5	20.4	23.3	26.2	29.1	32.0	35.0	37.9	40.8	43.7	46.6
NAD (µL)	1.7	2.5	1.7	2.1	2.5	2.9	3.3	3.7	4.1	4.5	5.0	5.4	5.8	6.2	6.6
DNA Prep Additive (Pre-diluted 1:5 in Enzyme Dilution Buffer) (µL)	1.7	2.5	1.7	2.1	2.5	2.9	3.3	3.7	4.1	4.5	5.0	5.4	5.8	6.2	6.6
DNA Prep Enzynme	1.7	2.5	1.7	2.1	2.5	2.9	3.3	3.7	4.1	4.5	5.0	5.4	5.8	6.2	6.6
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**
DNA Damage Repair Mix v2 (µL)	3.4	5.0	3.4	4.2	5.0	5.9	6.7	7.5	8.4	9.2	10.1	10.9	11.7	12.6	13.4
Enzyme Dilution Buffer (µL)	1.7	2.5	1.7	2.1	2.5	2.9	3.3	3.7	4.1	4.5	5.0	5.4	5.8	6.2	6.6
Table 3. Ligation Master Mix	**Vol. (µL)**														
Plex Level	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Ligation Mix (µL)	44	66	44	55	66	77	88	99	110	121	132	143	154	165	176
Ligation Additive (µL)	1.5	2.2	1.5	1.8	2.2	2.5	2.9	3.3	3.6	4.0	4.4	4.7	5.1	5.4	5.8
Ligation Enhancer (µL)	1.5	2.2	1.5	1.8	2.2	2.5	2.9	3.3	3.6	4.0	4.4	4.7	5.1	5.4	5.8

Revision History (Description)	Version	Date
Initial release.	01	February 2019