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®, Sequel II, and Sequel IIe 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.

The recommended multiplex level for this procedure is:

	Maximum Multiplex / SMRT® Cell		
PacBio System	**Microbes**	**Total Genome Size**	**Barcoded Adapters**
Sequel System	16	30 Mb	Barcoded Overhang Adapter Kit 8A (101-628-400) Barcoded Overhang Adapter Kit 8B (101-628-500)
Sequel II or IIe System	96	375 Mb	SMRTbell Barcoded Adapter Plate 3.0 (102-009-200)

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.

Required Materials

Item	Vendor	Part Number
DNA QC (One of the following)		
CHEF Mapper XA	Bio-Rad	170-3670
Pippin Pulse Electrophoresis Power Supply	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 3	Diagenode	B06010003
Megaruptor 3 Shearing Kit	Diagenode	E07010003
SMRTbell Library Preparation		
SMRTbell Express Template Prep Kit 2.0	PacBio	100-938-900
Barcoded Adapters	PacBio	Sequel II/IIe Systems: 102-009-200 Sequel System: 101-628-400 & 101-628-500
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

Table 1. List of Required Materials and Equipment.

Workflow

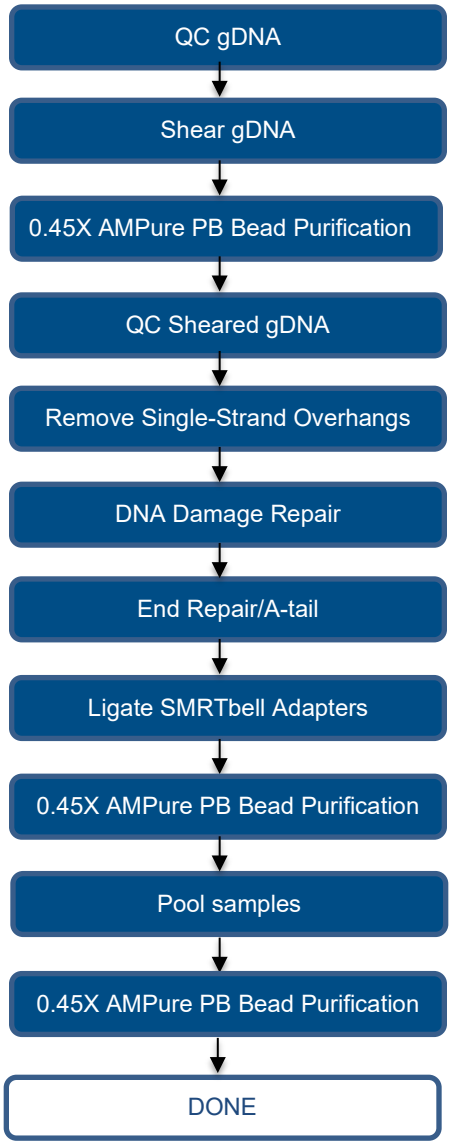

Figure 1: Workflow for preparing a multiplexed microbial library using the SMRTbell Express Template Prep Kit 2.0 and barcoded adapters.

Best Practices Recommendations

- Use a multi-channel pipette and a 96-well plate or 8-tube strips.
- For pipetting ≤2 µL, use a P2 pipette or a multi-channel pippete 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.
- Several tubes in the SMRTbell Express TPK 2.0 kit (shown in table 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 2: Temperature sensitive reagents

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 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 3. gDNA Quality QC Methods and Procedures.

Shear Genomic DNA to 7 kb - 10 kb

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 shearing, use the recommended concentration and volume as for the final shears.

Shear Genomic DNA Using 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. 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 3287 x g (7000 rpm in the Eppendorf MiniSpin Plus) for 2 minutes to achieve a target mode size of 7 kb – 10 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 3287 x g (7000 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 3

STEP	✓	Shear DNA	Notes
1		Dilute **1.0 µg** gDNA to **10 ng/µL** in Elution Buffer in a final sample volume of **100 µL**. Before shearing, we recommend removing a 1 µL aliquot (un-sheared sample) for sizing QC.	
2		Shear gDNA at a speed of 40 to achieve a target mode size of 7 kb – 10 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.	

Purify Sheared DNA Using 0.45X AMPure PB Beads

STEP	✓	Concentrate DNA	Notes
		Notes for proper handling of AMPure PB beads: – 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 until beads separate fully from the solution.	
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. – 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-Strand Overhangs”). – 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 37ºC 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.	

Quantify and Qualify 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 7 kb – 10 kb.

   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 1 µg per library. 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 enter the DNA sample concentration values measured in Step 13 of the 'Purify SMRTbell Library Using 0.45X AMPure® PB Beads Before Pooling' section 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

Remove Single-Strand Overhangs

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 37ºC for 15 minutes, then return the reaction to 4ºC. 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 37ºC 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 20ºC for 10 minutes, then 65°C for 30 minutes, then return the reaction to 4°C. Proceed to the next step.

Ligate SMRTbell Adapters

1. For ligation, the barcoded SMRTbell adapter must be added to the A-tailed sample first before adding ligase.

Reagent	Volume	✓	Notes
A-tailed sample	19.9 µL		
Barcoded Adapter	2.0 µL		
Total Volume	21.9 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. Let sit on ice and proceed to the next step.
4. 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.

5. 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		

6. 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.
7. Incubate at 20°C for 60 minutes.
8. Heat kill the ligase before proceeding to the sample pooling by incubating the samples at 65°C for 10 minutes.
9. Proceed to the next step.

Purify Using 0.45X AMPure PB Beads Before Pooling

Equimolar pooling is needed for even representation across samples. 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 beads to the ligation reaction.	
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 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		Wash beads with freshly prepared 80% ethanol. – 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		Remove residual 80% ethanol. – 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		Elute in **20 µL** 1x Elution buffer. Finger-tap the tube to mix until beads are uniformly re-suspended (for Lo-Bind microcentrifuge tubes and 8-tube strip). 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. – 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		Measure the DNA concentration using a Qubit fluorometer. – 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 250 µL for 32-plex, 350 µL for 48-plex.

Multiplex Level	Total Pool Volume (µL)
8	100
16	150
24	200
32	250
40	300
48	350

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 Using 0.45X AMPure PB Beads Before Pooling' section 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.

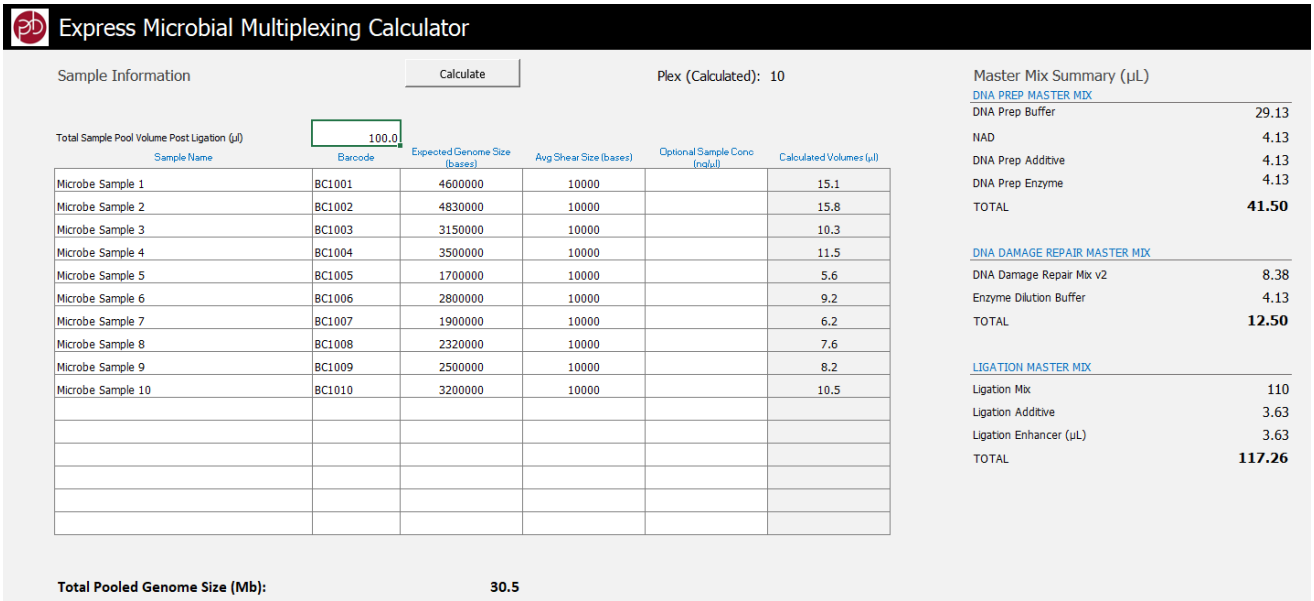

Figure 2. Microbial Multiplexing Calculator with 10-plex example.

AMPure PB Bead Purification of Pooled SMRTbell Libraries

STEP	✓	Purify DNA	Notes
		Notes for proper handling of AMPure PB beads: – Bring 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	
8		Wash beads with freshly prepared 80% ethanol. – 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. – 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 **11 µL** elution buffer to the beads to elute the DNA: – 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.	
13		Proceed to “Quantification and Qualification of Final Pooled SMRTbell Libraries”.	

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 are approximately 20%.
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).

Prepare for Sequencing

Follow the instructions in SMRT Link Sample Setup v10.2 or higher. Select “Microbial Assembly” from the application dropdown.

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
Added Multiplex level and corresponding pool volume to table on page 14.	06	March 2020
Updated table format on page 16 to clarify last bullet in step 8.	07	July 2020
Updated for HiFi-based assembly (7 kb -10 kb fragments) and SMRTbell Barcoded Adapter Plate 3.0.	08	November 2021