Preparing whole genome and metagenome libraries using SMRTbell® prep kit 3.0

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing whole genome sequencing (WGS) libraries from genomic and metagenomic DNA using the SMRTbell prep kit 3.0 for sequencing on PacBio® long-read systems.

Overview	
Libraries per SMRTbell prep kit 3.0	1–24
QC and workflow time (8 samples, manual preparation)	
• Genomic DNA QC on Femto Pulse	1.5 hours
• Library prep with SMRTbell prep kit 3.0	4 hours
• SMRTbell library QC on Femto Pulse	1.5 hours

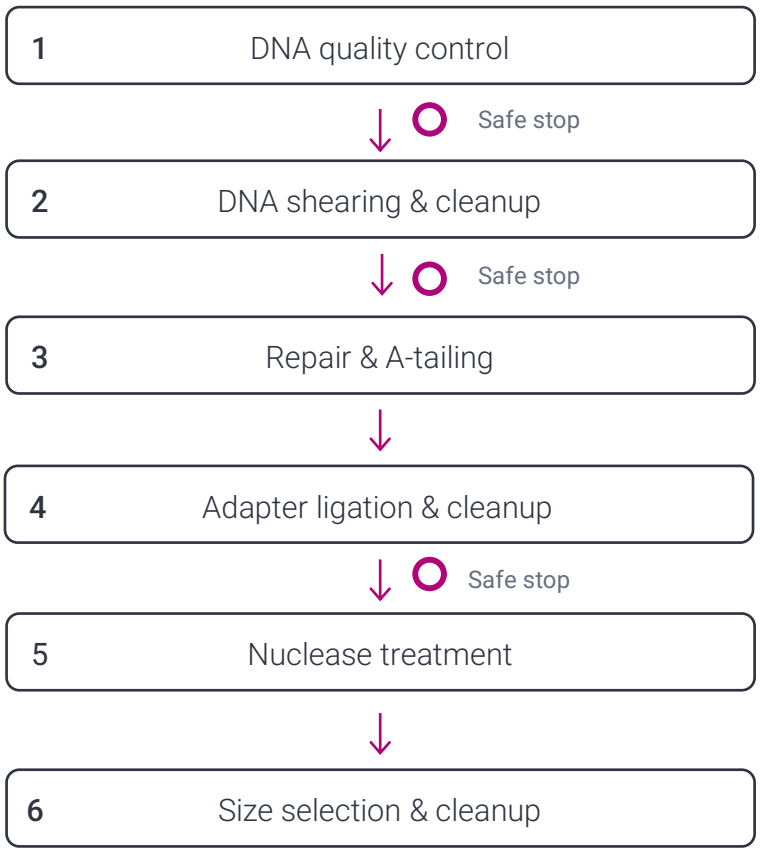
DNA input	Sequel II® and Sequel IIe	Revio™
Single or pooled library	1 µg per SMRT® Cell 8M	2 µg per Revio SMRT Cell
Multiplex libraries	300 ng – 1 µg per sample	300 ng – 2 µg per sample

DNA Quality	Human, plant, and animal samples	Microbial and Metagenomic samples
DNA size distribution (Femto Pulse system)	50% ≥30 kb & 90% ≥10 kb	90% ≥7 kb

DNA Shearing	Human, plant, and animal samples	Microbial and Metagenomic samples
DNA shearing (Megaruptor 3 system)	Speed 31	Speed 40
Target fragment lengths	15–18 kb	7–12 kb

102-166-600 REV02 MAR2023

Workflow overview



Required materials and equipment

PCR tube strips are recommended for the enzymatic and bead cleanup steps, but Eppendorf Lo-bind tubes or 0.2 mL 96-well PCR plates are also acceptable. No difference in performance is expected across PCR tube strips, Lo-bind tubes, or plates.

DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
DNA quantitation	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
DNA shearing	
Megaruptor 3 system	Diagenode B06010003
Megaruptor 3 shearing kit	Diagenode E07010003
SMRTbell® library preparation	
SMRTbell® prep kit 3.0	PacBio 102-182-700
SMRTbell® barcoded adapter plate 3.0 (optional; for barcoding)	PacBio 102-009-200
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
8-channel pipettes	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
Thermocycler compatible with 0.2 mL 8-tube strips	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021
Size selection	
AMPure® PB bead size selection kit	PacBio 102-182-500

General best practices

The following are general best practices for whole genome sequencing (WGS) using the Sequel II/IIe or Revio systems. Please read carefully prior to beginning library prep.

DNA quality

For human, animal, and plant genomes, the recommended Genome quality number (GQN) is 9.0 or higher at 10 kb and 5.0 or higher at 30 kb. High-quality genomic DNA will maximize sequencing coverage per SMRTbell library and reduce sequencing costs. For samples that do not meet these metrics, the SRE kit can be used to improve sample quality by depleting degraded DNA prior to shearing.

For prokaryotic genomes or metagenomic sequencing applications, the recommended GQN is 9.0 or higher at 7 kb.

Samples should be free of RNA before beginning library prep. If RNA is present, treat with RNase A and incubate at 37°C for 15 minutes. Clean reactions using a 1X concentration of SMRTbell cleanup beads (or AMPure PB) before proceeding. RNA can reduce loading of your library on the SMRT Cell if not removed prior to library prep.

DNA input

Note: Using lower than recommended DNA input amounts may result in insufficient SMRTbell library product for loading at optimal concentrations.

Increase per-SMRT Cell DNA input amounts by at least 50% when using a gel-cassette size selection option.

Each library prep reaction supports up to 5 µg of sheared genomic DNA.

As little as 300 ng of DNA input may be used per sample when multiplexing. However, the sum across all samples should be greater than the recommended minimal DNA amount per SMRT Cell (e.g., 1 µg for Sequel II/IIe, or 2 µg for Revio system). For example, if planning to multiplex 4 samples on a Revio SMRT Cell, use at least 500 ng of sheared DNA per sample.

Procedural details on additional size selection options can be found here: *Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0* .

DNA shearing

This protocol utilizes the Megaruptor 3 system for shearing. Please see *Technical Note - Covaris g-TUBE DNA shearing for SMRTbell prep kit 3.0* for an alternative shearing method.

Target shear lengths refer to the mean or modal size of the distribution. The recommended sizes allow for the best balance between read length and yield. Over-shearing DNA (small fragments) will result in lower genome coverage (less yield per HiFi read), while under-shearing (larger fragments >25 kb) may result in lower HiFi sequencing yields due to fewer HiFi reads.

Microbial and metagenomics samples may forgo shearing if the DNA is already in the specified fragment length range (7–12 kb). In such cases, clean the samples with a 1X concentration of SMRTbell cleanup beads as described in section 2 of the protocol.

Adjust shearing speeds or DNA concentration as necessary to produce the desired target fragment lengths.

Reagent and sample handling

Room temperature is defined as any temperature in the range of **18–23°C** for this protocol.

Thaw the repair buffer, nuclease buffer, and elution buffer at room temperature.

Mix reagent buffers with a brief vortex prior to use. Do not vortex enzymes.

Quick-spin all reagents in microcentrifuge to collect liquid at bottom prior to use.

Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Repair and A-tailing	Blue	End repair mix
	Green	DNA repair mix
Adapter ligation	Orange	SMRTbell adapter
	Yellow	Ligation mix
	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix

Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.

It is recommended to pipette-mix all SMRTbell prep reactions by pipetting up and down 10 times.

Samples can be stored at 4°C at all safe stopping points listed in the protocol.

Multiplexing recommendations for long-read sequencing

Barcode libraries using the SMRTbell barcoded adapter plate 3.0.

Standard multiplexing recommendations for WGS:

1. Shear all samples to similar fragment length profiles and verify on the Agilent Femto Pulse system. Note: the Agilent Fragment Analyzer can be used for shearing QC in high throughput applications.
2. Prepare libraries with SMRTbell prep kit 3.0 and SMRTbell barcoded adapters.
3. Pool samples with similar genome sizes together.
4. Pool an equal mass of each SMRTbell library into the pool.

These recommendations help balance coverage across samples in the pool. Both the number of reads (molarity) and insert size of the libraries (mass) influence the yield per library, while genome size impacts how yield translates to coverage.

If fragment length profiles are similar but the estimated genome sizes of the samples are different, pool the libraries with mass proportional to genome size. For example, if pooling a 2 Gb genome sample with a 1 Gb genome sample, add twice the mass of library for the 2 Gb genome sample relative to the 1 Gb genome sample.

Pooling libraries with different fragment length profiles

When the difference in average length of your SMRTbell libraries is greater than 25%, it may be necessary to pool in equal molar amounts to balance the number of reads per library. Please note, this will help balance the number of reads, but is unlikely to balance sequence coverage due to the different insert length profiles of the libraries.

To pool samples in equal molarity:

1. Match the molarity of all SMRTbell libraries.
    1.1 Measure the concentration of each library on a Qubit fluorometer using the dsDNA HS assay.
    1.2 QC samples on the Agilent Femto Pulse system and use a smear analysis to determine the average length of each SMRTbell library
    1.3 Use the SMRT Link conversion calculator in Sample Setup (or other tool) to convert concentration and size to molarity for each SMRTbell library
    1.4 Select a final molarity and volume to use for the final pool. Typically, use the lowest molarity library, but samples should be >1.7 nM or >20 ng/µL.
    1.5 Adjust the molarity of each SMRTbell library to the target molarity (above) with PacBio elution buffer (EB). If pooling samples with different genome sizes, adjust the molarity proportional to the genome size. Please note that this may not always be possible depending on the starting molarity of the sample and the molarity of the smallest genome in the pool.
2. Pool by combining equal volumes of each normalized SMRTbell library. Total volume should equal the volume selected in step 1.4.

Thermocycler programs

Program thermocycler(s) prior to beginning the protocol for the first time.

Repair and A-tailing, adapter ligation, and nuclease treatment thermocycler steps can be combined into a single program and paused in between prep treatments if preferred.

Set the lid temperature to 75°C for all programs. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

1. Repair and A-tailing program

Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

2. Adapter ligation program

Step	Time	Temperature
1	30 min	20°C
2	Hold	4°C

3. Nuclease treatment program

Step	Time	Temperature
1	15 min	37°C
2	Hold	4°C

Workflow steps

1. DNA quality control

Evaluate the quantity and size distribution of input DNA to determine whether it is suitable for this protocol.

✔	Step	Instructions for genomic DNA QC
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to **room temperature**.
	1.2	Pulse vortex or pipette-mix each sample to homogenize the DNA in solution.
	1.3	Quick-spin each sample to collect liquid.
	1.4	Take a **1 µL** aliquot from each sample and dilute with **9 µL** of **elution buffer or water.**
	1.5	Measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.**
	1.6	Dilute each aliquot to **250 pg/µL** in Femto Pulse dilution buffer based on the Qubit reading.
	1.7	Measure DNA size distribution with a Femto Pulse system using the **gDNA 165kb analysis kit.**
	1.8	Proceed to the next step of the protocol if sample quality is acceptable.
		SAFE STOPPING POINT - Store at 4°C

2. DNA shearing and cleanup

This step shears and concentrates the DNA to the appropriate size range.

✔	Step	Instructions DNA shearing and cleanup
		DNA shearing
	2.1	Bring DNA up to a final volume of **100–130 µL** with **low TE buffer**. Target a concentration of **30 ng/µL** (range: **3–39 ng/µL**).
	2.2	Shear DNA on the Megaruptor 3 system. Recommended settings are below.

Genome	Shear speed	Target insert length
Human, plant, or animal	**31**	15–18 kb
Microbe	**40**	7–10 kb

	2.3	Transfer sheared DNA into a tube strip or other appropriate tube for the 1x SMRTbell cleanup bead step. Typical volume loss during shearing is between 5–10 µL.
		Cleanup with 1X SMRTbell cleanup beads
	2.4	Add **1.0X** v/v (volume over volume) of resuspended, room-temperature SMRTbell cleanup beads to each tube of sheared DNA.
	2.5	Pipette-mix the beads until evenly distributed.
	2.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	2.7	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	2.8	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

✔	Step	Instructions DNA shearing and cleanup
	2.9	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	2.10	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	2.11	Repeat the previous step.
	2.12	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	2.13	Remove the tube strip from the magnetic rack. **Immediately** add **47 µL** of **low TE buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	2.14	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	2.15	Leave at **room temperature** for **5 minutes** to elute DNA.
	2.16	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	2.17	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	2.18	Recommended: Evaluate sample quality (concentration and size distribution). • Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute each aliquot to **250 pg/µL** in Femto Pulse dilution buffer. • Measure DNA size distribution with a Femto Pulse system.
	2.19	Proceed to the next step of the protocol if sample quality is acceptable.

SAFE STOPPING POINT - Store at 4°C

3. Repair and A-tailing

This step repairs sites of DNA damage and prepares the sheared DNA for ligation to the SMRTbell adapter.

✔	Step	Instructions for DNA damage and end repair

3.1 Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. If preparing 8 or more libraries, increase the overage to 25% to ensure there is enough reaction mix to dispense. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip **RM1** steps (3.2 to 3.4).

Reaction Mix 1 (RM1)

✔	Tube	Component	Volume		
			Per library	4 libraries	8 libraries
	Purple	Repair buffer	8 µL	35.2 µL	80 µL
	Blue	End repair mix	4 µL	17.6 µL	40 µL
	Green	DNA repair mix	2 µL	8.8 µL	20 µL
		Total volume	**14 µL**	**61.6 µL**	**140 µL**

3.2 Pipette-mix **RM1**.

3.3 Quick-spin **RM1** in a microcentrifuge to collect liquid.

3.4 Add **14 µL** of the **RM1** to each sample**.** Total reaction volume should be **60 µL**.

3.5 Pipette-mix each sample.

3.6 Quick-spin the tube strip in a microcentrifuge to collect liquid.

3.7 Run the **repair and A-tailing** thermocycler program.

Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

3.8 Proceed to the next step of the protocol.

4. Adapter ligation and cleanup

This step ligates the SMRTbell adapter to the ends of each DNA fragment.

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
		Adapter ligation
	4.1	If barcoding samples: add **4 µL** of barcoded adapters from the **SMRTbell barcoded adapter plate 3.0** to each respective sample from the previous step and exclude the SMRTbell adapter from Reaction Mix 2. Skip this step if not barcoding samples.
	4.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. If preparing 8 or more libraries, increase the overage to 25% to ensure there is enough reaction mix to dispense. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip RM2 steps (4.3 to 4.5).
	4.3	Pipette-mix **RM2**.
	4.4	Quick-spin **RM2** in a microcentrifuge to collect liquid.
	4.5	**Barcoded samples**: add **31 µL** of **RM2** to each sample from the previous step. **Non-barcoded samples:** add **35 µL** of **RM2** to each sample from the previous step. Total volume per sample should be **95 µL.**
	4.6	Pipette-mix each sample.
	4.7	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	4.8	Run the adapter ligation thermocycler program.
		Cleanup with 1X SMRTbell cleanup beads
	4.9	Add **95 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	4.10	Pipette-mix the beads until evenly distributed.
	4.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	4.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	4.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume		
			Per library	4 libraries	8 libraries
	Orange	SMRTbell adapter*	4 µL	17.6 µL	40 µL
	Yellow	Ligation mix	30 µL	132 µL	300 µL
	Red	Ligation enhancer	1 µL	4.4 µL	10 µL
		Total volume	**35 µL**	**154 µL**	**350 µL**

*Exclude the SMRTbell adapter if using the SMRTbell barcoded adapter plate 3.0.

Step	Time	Temperature
1	30 min	20°C
2	Hold	4°C

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
	4.15	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	4.16	Repeat the previous step.
	4.17	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	4.18	Remove the tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads.
	4.19	Quick-spin the tube strip in a microcentrifuge.
	4.20	Leave at **room temperature** for **5 minutes** to elute DNA.
	4.21	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
	4.23	Proceed to the next step of the protocol.

SAFE STOPPING POINT - Store at 4°C

5. Nuclease treatment

This step removes unligated DNA fragments and leftover SMRTbell adapter from the sample.

✔	Step	Instructions for nuclease treatment
	5.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. If preparing 8 or more libraries, increase the overage to 25% to ensure there is enough reaction mix to dispense. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip RM3 steps (5.2 to 5.4).
	5.2	Pipette-mix **RM3.**
	5.3	Quick-spin **RM3** in a microcentrifuge to collect liquid.
	5.4	Add **10 µL** of **RM3** to each sample. Total volume should equal **50 µL.**
	5.5	Pipette-mix each sample.
	5.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume		
			Per library	4 libraries	8 libraries
	Light purple	Nuclease buffer	5 µL	22 µL	50 µL
	Light green	Nuclease mix	5 µL	22 µL	50 µL
		Total volume	**10 µL**	**44 µL**	**100 µL**

5.7 Run the **nuclease treatment** thermocycler program.

Step	Time	Temperature
1	15 min	37°C
2	Hold	4°C

5.8 Proceed to the next step of the protocol.

Note: it is necessary to remove the nucleases using either AMPure PB size selection or SMRTbell cleanup beads prior to safely storing the library or libraries.

6. AMPure PB bead size selection or cleanup with SMRTbell cleanup beads

The AMPure PB bead size selection step will clean the library and deplete DNA fragments shorter than 5 kb. Please see the Technical note – HiFi WGS Performance with AMPure PB bead size selection for more information on performance of this method.

To remove SMRTbell molecules with inserts greater than 5 kb from the SMRTbell library, please see the Technical Note - Alternative size selection methods for SMRTbell prep kit 3.0. If performing an alternative method, or skipping size selection altogether, proceed to the bead binding, wash, and elution steps by adding a 1X concentration of the SMRTbell cleanup beads to each library (50 µL).

Diluting AMPure PB beads for size selection <5 kb

Size selection performance is sensitive to bead concentrations; therefore, ensure accurate pipetting volumes when diluting the beads and adding them to the library.

✔	Step	Instructions for AMPure PB bead size selection
	1	Make a 35% v/v dilution of AMPure PB beads by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of elution buffer. The 35% dilution can be stored at 4°C for 30 days. Note: the AMPure PB dilution may be scaled down as appropriate for smaller scale projects.
	2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	3	Proceed to **step 6.1** in the table below for bead binding, wash, and elution steps.

Bead binding, wash, and elution steps.

✔	Step	Instructions for bead binding, washing, and sample elution
	6.0	If **not** performing the diluted (35% v/v) AMPure PB bead size selection procedure, add **50 µL** of SMRTbell cleanup beads to each nuclease treated library from the previous step and proceed to step 6.1 below.
	6.1	Pipette-mix the beads until evenly distributed.
	6.2	Quick-spin the tube strip in a microcentrifuge to collect all liquid.

6.3	Leave at **room temperature** for **20 minutes** for AMPure PB bead size selection and **10 minutes** for SMRTbell cleanup beads to allow DNA to bind beads.
6.4	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
6.5	Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.
6.6	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
6.7	Repeat the previous step.
6.8	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick-spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
6.9	Remove tube strip from the magnetic rack. **Immediately** add **15 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
6.10	Quick-spin the tube strip in a microcentrifuge to collect liquid.
6.11	Leave at **room temperature** for **5 minutes** to elute DNA.
6.12	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
6.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
6.14	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution buffer or water**. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. Recommended: Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
6.15	Proceed to use SMRT Link Sample Setup to prepare library or libraries for sequencing. If libraries will be multiplexed, pool libraries as described in the multiplexing section under *General best practices*. The recommended Sample Setup input concentration range for WGS libraries is 20 to 60 ng/µL. When preparing multiple libraries for sequencing it may be necessary to normalize the concentration of the batch. If after normalization, or if preparing a single library, the concentration is > 60 ng/µL, then dilute the library or libraries with elution buffer to within the 20 to 60 ng/µL range before annealing the sequencing primer. Note: libraries that are below 20 ng/µL may not contain enough material (dependent on insert size) to load at the optimal concentration or for multiple SMRT Cells.
6.16	Store SMRTbell libraries at 4°C if sequencing within 1 week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release.	01	April 2022
Updated to include new information for the Revio system	02	March 2023