Preparing multiplexed AAV SMRTbell® libraries using SMRTbell prep kit 3.0

Procedure & checklist

Before you begin

This protocol describes how to prepare multiplexed AAV libraries for sequencing on the Sequel® II/IIe and Revio™ systems. Multiplexing achieved using SMRTbell Barcoded adapters.

Once barcoded, samples can be pooled and purified for sequencing on the Sequel II/IIe and Revio systems. A total of 1 µg of pooled AAV DNA is required for SMRTbell library preparation per SMRT® Cell. The input requirements per individual AAV sample depends on the multiplex level and can range from 250 ng for a 4-plex, to 42 ng per sample for a 24-plex preparation.

Preparation of AAV DNA for SMRTbell library preparation

AAV DNA may have several structures or formats: single-stranded DNA (ss), double-stranded DNA with some non-complementary regions (ds), or single-stranded self-complementary (sc) DNA, with an inverted repeat in the middle. All structures generally have inverted terminal repeats (ITR) at the ends.

SMRTbell template formation requires double-stranded DNA with blunt ends onto which hairpin SMRTbell adapters can be ligated, following A-tailing. Creating appropriate ds input DNA requires extra steps if the sample contains a substantial ss region without ds blunt ends, as shown on the left in Figure 1, below.

If the sample contains self-complementary molecules that have only one site accessible for hairpin adapter ligation, as shown on the right in Figure 1, it is important to collect and analyze data appropriately. Non-complementary ds molecules also require additional analysis.

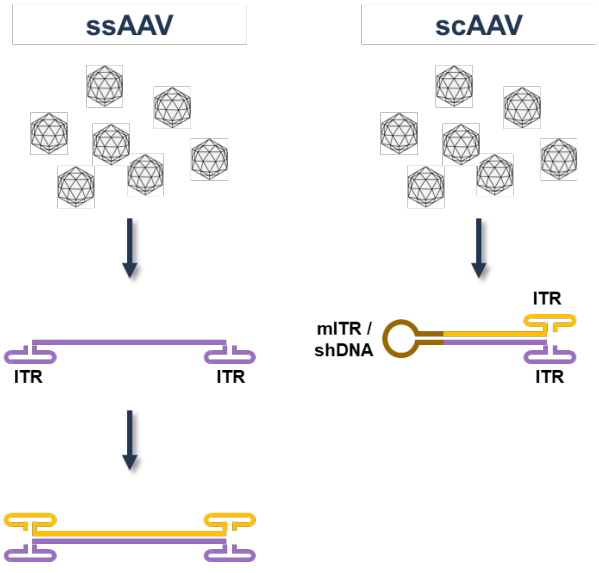

Figure 1: Structures of ss (single-stranded) and sc (self-complementary) DNA molecules. The ssDNA structure at left requires a second strand (as shown in yellow) for SMRTbell formation.

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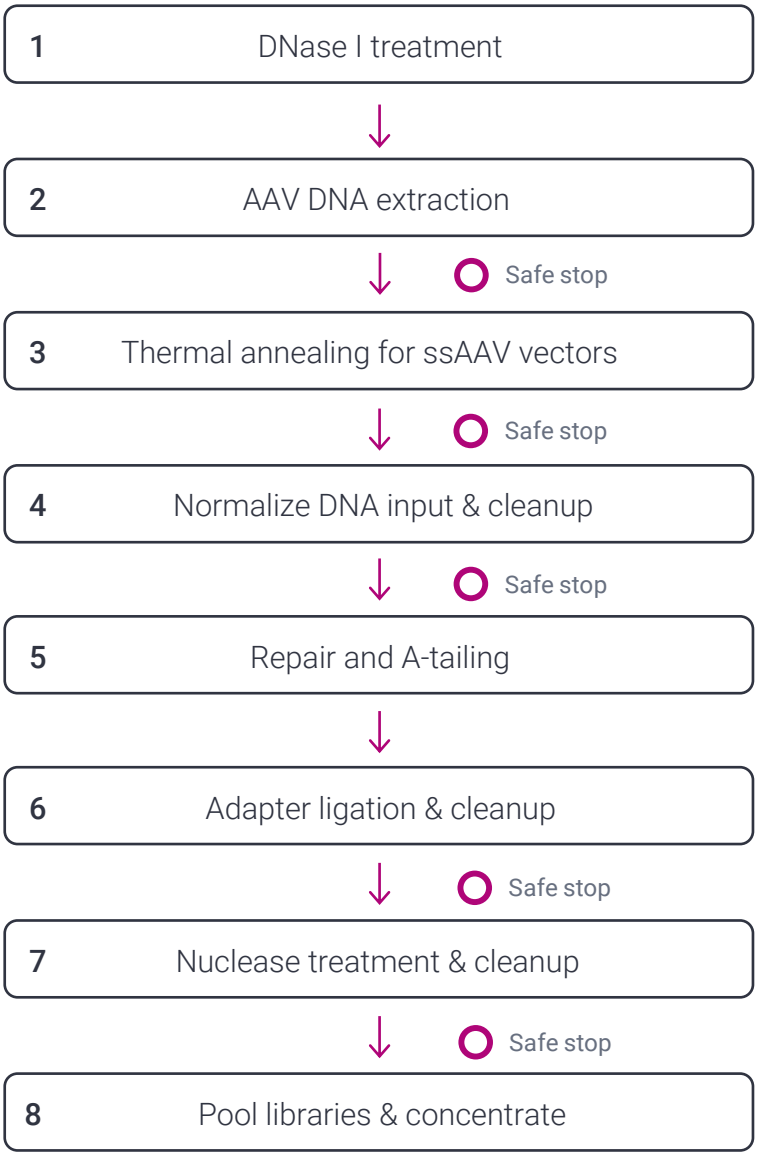
Workflow



Required materials and equipment

AAV DNA preparation	
PureLinkTM V\viral RNA/DNA mini kit	Thermo Fisher Scientific 12-280-050
DNase I (RNase-free)	NEB M0303S
Nuclease-free water, molecular biology grade	Any major lab supplier (MLS)
5M NaCl	Any MLS
1 M Tris-HCl [pH 8.5]	Any MLS
500 mM EDTA [pH 8]	Any MLS
DNA Quantification	
Qubit fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	Thermo Fisher Scientific Q33230
SMRTbell library preparation	
SMRTbell® prep kit 3.0	PacBio 102-182-700
SMRTbell® barcoded adapter plate 3.0	PacBio 102-009-200
Lab supplies and equipment	
8- or 12-Multi-channel Pipette	Any MLS
Single channel pipettes 1 to 1000 µL	Any MLS
0.2 mL PCR 8-tube strips	Any MLS
96 well plate (optional)	Any MLS
1.5 mL DNA LoBind® Tubes	Eppendorf 022431021
200 proof ethanol, molecular biology or ACS grade	Any MLS
Microcentrifuge	Any MLS
Magnetic separation rack for 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
DynaMag-2-magnet	Thermo Fisher Scientific 12321D
Thermocycler compatible with 0.2 mL strip tubes	Any MLS
2100 Bioanalyzer	Agilent Technologies, Inc. G2939BA
High sensitivity DNA kit	Agilent Technologies, Inc. 5067-4626

General best practices

AAV vector specific recommendations

Thermal annealing is required only for ssAAV templates. Prepare the 1X annealing buffer (25 mM NaCl, 10 mM Tris-HCl [pH 8.5], 0.5 mM EDTA [pH 8]) prior to the thermal annealing step.

Begin protocol at the Normalize DNA input & cleanup step (section 4) when starting with previously isolated AAV DNA.

Use a combined total of ≥**1 µg of AAV DNA per SMRT Cell 8M**. This is the combined total of samples that will be multiplexed. Per sample amounts should be **1 µg/number of samples**. An example of per sample input amounts are shown in the table below.

Multiplex level	DNA Input
4-plex	250 ng per sample
8-plex	125 ng per sample
12-plex	83 ng per sample
16-plex	63 ng per sample
24-plex	42 ng per sample

SMRTbell prep kit 3.0 reagent 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 and SMRTbell adapter with a brief vortex prior to use. Enzyme mixes do not require vortexing.

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 barcoded adapter plate 3.0
	Yellow	Ligation mix
	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix

Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30–60 minutes prior to use.

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

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

Barcode with SMRTbell Barcoded Adapter Plate 3.0. Quick-spin the plate to collect liquid at bottom of the well prior to use.

Pooling libraries that have similar average insert sizes will provide more even sequence coverage between samples. Increasing differences in average molecular weights will lead to increasing differences in coverage across libraries because of differences in molarity for SMRT Cell loading. For sensitive experiments where balanced coverage is critical, consider pooling libraries in equal molar amounts.

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 & 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

Procedure & checklist

1. DNase I treatment

Treat sc / ss AAV vector with DNase I to remove the non-encapsidated DNA from the vector. The capsid shell is resistant to DNase I treatment, therefore, DNase I will not degrade the encapsidated DNA.

✔	Step	Instructions
	1.1	Prepare the DNase I reaction mix as per the table below. Example showing addition of 20 units of DNase I to 7E11 vector genomes in a 200 µL volume.

Reagent	Volume (µL)
AAV vector	50
Nuclease-free Water	120
Reaction Buffer	20
DNase I (RNase-free)	10
Total Volume	**200**

	1.2	Pipette-mix.
	1.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	1.4	Incubate at 37°C for 10 minutes.
	1.5	Proceed to the next step of the protocol.

2. AAV DNA extraction

Extract DNA from DNase I treated sc / ss AAV vector by using PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions. This kit removes fragments <200 bp. Therefore, if the AAV sample contains fragments <200 bp, it is advised to use alternative extraction methods such as Phenol/chloroform/isoamyl alcohol with Proteinase treatment (**reference**).

3. Thermal annealing for ssAAV vectors

For ssAAV vectors, thermal annealing is performed to anneal the (+) and (-) strands of the AAV vector and form a double-stranded structure for SMRT® sequencing. Follow the annealing procedure below. **Skip section if working with scAAV vectors.**

✔	Step	Instructions
	3.1	Resuspend ssAAV DNA in 1X annealing buffer in a 1 to 10 ratio (ssAAV DNA to annealing buffer). For example, if the elution volume of the ssAAV sample is 10 µL, then add 90 µL of 1X annealing buffer (25 mM NaCl, 10 mM Tris-HCl [pH 8.5], 0.5 mM EDTA [pH 8]).
	3.2	Incubate the tube at **95°C** for **5 min** and then ramp down to **25°C** (**1 min for every −1°C**) on a thermocycler.
	3.3	Use **1 µL** of the annealed sample and make a 1:5 dilution in elution buffer.
	3.4	Use **1 µL** of this 1:5 dilution to measure the DNA concentration using the Qubit dsDNA HS Assay kit according to the manufacturer's recommendations.
	3.5	Dilute **1 µL** of sample to **1.5 ng/µL** and run **1 µL** on an Agilent bioanalyzer system using a High sensitivity DNA kit.
	3.6	Proceed to the next step of the protocol.
		SAFE STOPPING POINT

4. Normalize DNA input & cleanup

✔	Step	Instructions
		Normalize DNA
	4.1	Determine the per AAV vector sample amount to use by dividing **1 µg** by the number of samples to be multiplexed. Use an equal mass of DNA for each vector that will multiplexed. The total amount of DNA going into SMRTbell library prep should be **1 µg**.
	4.2	Add the appropriate volume that equals the mass determined in the previous step to each respective 0.2 mL PCR strip tube. Bring all samples up to 86 µL with nuclease-free water or elution buffer.
		Cleanup with 1.3X SMRTbell cleanup beads
	4.3	Add **1.3X volume over volume (v/v) (112 mL)** of resuspended, room-temperature SMRTbell cleanup beads to each tube.
	4.4	Pipette-mix the beads until evenly distributed.
	4.5	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	4.6	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	4.7	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
	4.8	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	4.9	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.
	4.10	Repeat the previous step.
		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.

4.11	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 until evenly distributed.
4.12	Quick-spin the tube strip in a microcentrifuge to collect liquid.
4.13	Leave at **room temperature** for **5 minutes** to elute DNA.
4.15	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
4.16	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard the old tube strip with beads.
4.17	Proceed to next step or store samples at 4°C if stopping.

SAFE STOPPING POINT – Store at 4°C

5. Repair and a-tailing

✔	Step	Instructions
	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 samples being prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip RM1 steps (5.2 to 5.4).

✔	Tube	Reagent	Volume
	Previous	Contents from previous step	46 µL
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	**60 µL**

5.2	Pipette-mix **RM1**.
5.3	Quick-spin **RM1** in a microcentrifuge to collect liquid.
5.4	Add **14 µL** of the **RM1** to each sample. Total reaction volume should be **60 µL**.
5.5	Pipette-mix each sample.
5.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.
5.7	Run the **repair & A-tailing** thermocycler program.
5.8	Proceed to the next step of the protocol.

6. Adapter ligation

✔	Step	Instructions
		Adapter ligation
	6.1	Add **4 µL** of the SMRTbell barcoded adapter 3.0 to each sample from the previous step.
	6.2	Add the following components in the order and volume listed below to a fresh microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip RM2 steps (6.3 to 6.5).
	6.3	Pipette-mix **RM2**.
	6.4	Quick-spin **RM2** in a microcentrifuge to collect liquid.
	6.5	Add **31 µL** of **RM2** to each sample from previous step. Total volume should be **95 µL.**
	6.6	Pipette-mix each sample.
	6.7	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	6.8	Run the **adapter ligation** thermocycler program.
		Cleanup with 1.3X SMRTbell cleanup beads
	6.9	Add **124 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.10	Pipette-mix the beads until evenly distributed.
	6.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	6.12	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	6.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.15	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.
	6.16	Repeat the previous step.
	6.17	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.
	6.18	Remove the tube strip from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to each tube and resuspend the beads.
	6.19	Quick-spin the tube strip in a microcentrifuge.

Reaction Mix 2 (RM2)

✔	Tube	Component	Volume per Sample
	Yellow	Ligation mix	30 µL
	Red	Ligation enhancer	1 µL
		Total volume	**31 µL**

6.20	Leave at **room temperature** for **5 minutes** to elute DNA.
6.21	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
6.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new tube strip**. Discard old tube strip with beads.
6.23	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4 °C

7. Nuclease treatment & cleanup

✔	Step	Instructions
		Nuclease treatment
	7.1	Add the following components in the order and volume listed below to a microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below, then skip **RM3** steps (7.2 to 7.4).

Reaction Mix 3 (RM3)

✔	Tube	Component	Volume per sample
	Light purple	Nuclease Buffer	5 µL
	Light green	Nuclease Mix	5 µL
		Total Volume	**10 µL**

7.2	Pipette-mix **RM3**.
7.3	Quick-spin **RM3** in a microcentrifuge to collect liquid.
7.4	Add 10 µL of **RM3** to each sample. Total volume should equal 50 µL**.**
7.5	Pipette-mix each sample.
7.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.
7.7	Run the **nuclease treatment** thermocycler program.
	Cleanup with 1.3X SMRTbell cleanup beads
7.8	Add **65 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
7.9	Pipette-mix the beads until evenly distributed
7.10	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tube.
7.11	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
7.12	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
7.13	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
7.14	Slowly dispense **200 µL,** or enough **freshly prepared 80% ethanol** to cover the beads into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.

	7.15	Repeat the previous step.
	7.16	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.
	7.17	Remove the tube strip from the magnetic rack. **Immediately** add **12 µL** of **elution buffer** and resuspend the beads by pipetting 10 times or until evenly distributed.
	7.18	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	7.19	Leave at **room temperature** for **5 minutes** to elute DNA.
	7.20	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	7.21	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new strip tube**. Discard old tubes with beads.
	7.22	OPTIONAL: Take **1 µL** and measure DNA concentration with a Qubit fluorometer system using the 1x dsDNA HS kit to check for variable sample loss prior to pooling barcoded samples.

8. Pool barcoded AAV samples & concentrate

✔	Step	Instructions
	8.1	Pool together the entire elution from the previous step for each barcoded AAV sample into the same 1.5 mL DNA loBind tube.
	8.2	Add **1.3X v/v** of resuspended, room-temperature SMRTbell cleanup beads.
	8.3	Pipette-mix the beads until evenly distributed.
	8.4	Quick-spin the tube in a microcentrifuge to collect all liquid from the sides.
	8.5	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	8.6	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	8.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	8.8	Slowly dispense **200 µL,** or enough **freshly prepared 80% ethanol** to cover the beads into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	8.9	Repeat the previous step.
	8.10	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	8.11	Remove the tube from the magnetic rack. **Immediately** add **12 µL** of **elution buffer** and resuspend the beads by pipetting 10 times or until evenly distributed.
	8.12	Quick-spin the tube in a microcentrifuge to collect liquid.

8.13	Leave at **room temperature** for **5 minutes** to elute DNA.
8.14	Place the tube in a magnetic separation rack until beads separate fully from the solution.
8.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 1.5 mL DNA LoBind tube. Discard old tube with beads.
8.16	Evaluate SMRTbell library concentration and size distribution. • Take a **1 µL** aliquot and dilute with **9 µL** of **elution buffer or water**. • Measure DNA concentration with a Qubit Fluorometer system using the 1x dsDNA HS kit. • Measure the SMRTbell library size distribution with the 2100 Bioanalyzer system using the High Sensitivity DNA Kit.
8.17	Proceed to SMRT Link Sample Setup to prepare samples for sequencing.
8.18	Store SMRTbell libraries at **4°C** if sequencing within the 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
On page 4, updated total amount of AAV DNA to 1 µg.	02	April 2022
Updated to include compatibility with the Revio system	03	January 2024