Preparing multiplexed AAV SMRTbell® libraries using SMRTbell prep kit 3.0

Procedure & checklist

Before you begin

This protocol describes how to prepare multiplexed Adeno-associated virus (AAV) libraries for sequencing on PacBio® HiFi systems.

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. 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.

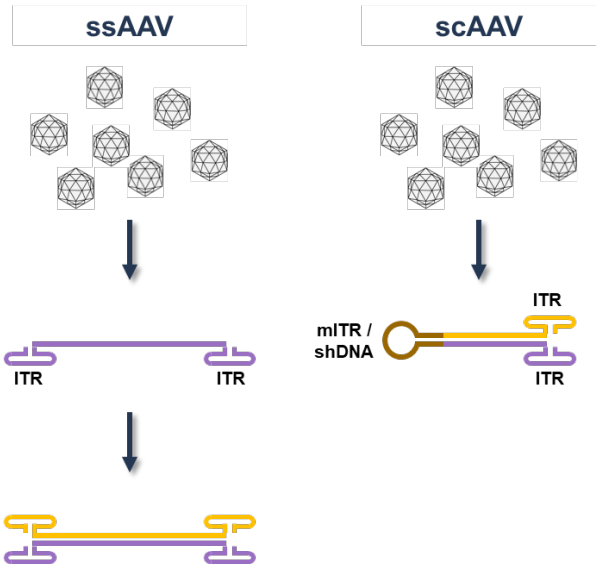

Figure 1: Structures of ss (single-stranded) and sc (self-complementary) DNA molecules. The ssDNA structure at left requires a second strand (as shown in yellow) for SMRTbell formation.

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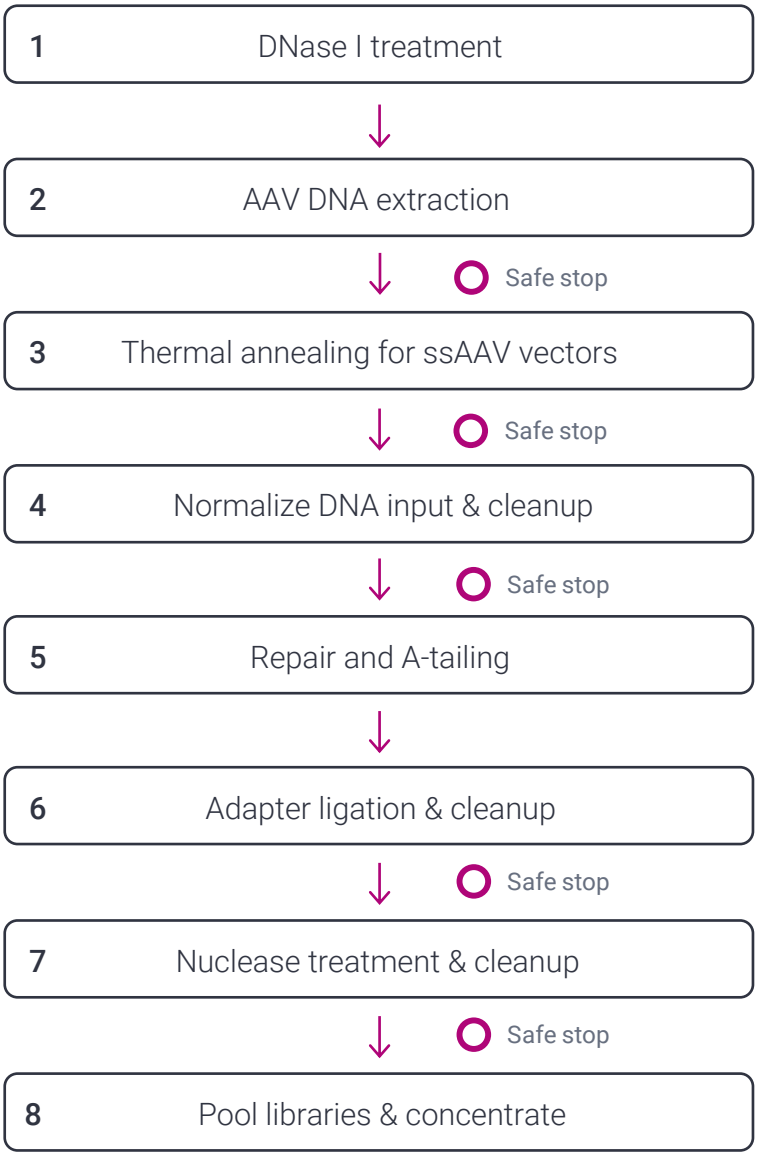
Workflow



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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 • SMRTbell® prep kit 3.0 • SMRTbell® cleanup beads • Low TE buffer	PacBio® 102-182-700
SMRTbell® barcoded adapter plate 3.0	PacBio® 102-009-200
Revio SPRQ™ polymerase kit *or* Vega™ polymerase kit *or* Sequel® II binding kit 3.2*	PacBio® 103-496-900 PacBio® 103-426-500 PacBio® 102-194-100
* Procedure for Sequel II binding kit 3.2 can be found in SMRT® Link Sample Setup	
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

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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.

See Table 1 below for guidance on DNA input mass requirements to yield enough final bound library for optimal loading on the appropriate sequencer. If multiplexing, divide the total recommended DNA input mass by the sample plex, as shown below.

Table 1. SMRTbell prep kit 3.0 per sample mass when multiplexing.

Sequencer	Total DNA Input mass per SMRT Cell	4-plex	8-plex	16-plex	24-plex	48-plex
Sequel II/e	1000 ng	250 ng	125 ng	63 ng	42 ng	Not recommended
Vega	2000 ng	500 ng	250 ng	125 ng	83 ng	Not recommended
Revio	2000 ng	500 ng	250 ng	125 ng	83 ng	42 ng
Revio +SPRQ/SPRQ-Nx	500 ng	125 ng	63 ng	31 ng	20 ng	20 ng*

* a mass of less than 20 ng into SPK3.0 is not recommended

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 SMRTbell adapter at room temperature. Mix reagent buffers with a brief vortex prior to use. Place on ice.

Thaw the Elution buffer. This can be stored at room temperature.

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

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	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.

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

Anneal, bind, and cleanup using the Revio or Vega polymerase kit

Thaw the following reagents at room temperature:

Component	Tube color
Annealing buffer	Light blue
Standard sequencing primer	Light green
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue

Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on ice. The Loading buffer should be left at room temperature.

Note that the Loading buffer is light sensitive and should be protected from light when not in use.

The sequencing polymerase and the sequencing control should kept on a cold block or ice. Bring the Loading buffer to room temperature 30 minutes prior to use.

Multiplexing

Barcode with SMRTbell barcoded adapter plate 3.0. Quick-spin the plate to collect liquid at bottom of the well prior to use.

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 at 4°C in between prep treatments if preferred. The lid temperature should be set to at least 10 °C above the incubation temperature.

1. Repair & A-tailing program

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

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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

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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 (**Tran et al., 2020**).

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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 mass to use by dividing **the required mass (see Table 1)** by the number of samples to be multiplexed. Use an equal mass of DNA for each vector that will multiplexed.
	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 µL)** 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	Incubate 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 remove 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**, remove 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. • Remove residual 80% ethanol and discard.

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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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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 to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 15% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip Repair master mix steps 5.2 to 5.4.

Repair master mix

✔	Tube	Reagent	Volume
	Purple	Repair buffer	8 µL
	Blue	End repair mix	4 µL
	Green	DNA repair mix	2 µL
		Total volume	14 µL

5.2	Pipette-mix **Repair master mix**.
5.3	Quick-spin **Repair master mix** in a microcentrifuge to collect liquid.
5.4	Add **14 µL** of the **Repair master mix** to sample from step 4.16 (46 µl) for a total reaction volume of **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 with the lid temperature set to >75°C.

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

5.8	Proceed to the next step of the protocol.

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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 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, and skip Ligation master mix steps 6.3 to 6.5.

Ligation master mix

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

	6.3	Pipette-mix **Ligation master mix**.
	6.4	Quick-spin **Ligation master mix** in a microcentrifuge to collect liquid.
	6.5	Add **31 µL** of **Ligation master mix** to each sample from step 5.7 (60 µL) for a total volume of **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 with the lid temperature set to >30°C.

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

		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	Incubate 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 remove 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**, remove the 80% ethanol and discard.
	6.16	Repeat the previous step.

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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. • Remove 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.
6.20	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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 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, and skip **Nuclease master mix** steps 7.2 to 7.4.

Nuclease master mix

✔	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 **Nuclease master mix**.
7.3	Quick-spin **Nuclease master mix** in a microcentrifuge to collect liquid.
7.4	Add 10 µL of **Nuclease master mix** 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

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	7.10	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tube.
	7.11	Incubate 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 remove 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**, remove 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. • Remove 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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	Incubate 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 remove 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**, remove the 80% ethanol and discard.
	8.9	Repeat the previous step.

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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. • Remove residual 80% ethanol and discard.
8.11	Remove the tube from the magnetic rack. **Immediately** add **26 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	Incubate 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 aspirate the cleared eluate without disturbing the beads. Transfer eluate 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	If needed, dilute **25 µL of library to <20 ng/µL**. Failure to normalize libraries or pools of libraries to the appropriate concentration prior to ABC may result in low sequencing yield.
8.18	Proceed to Section 9 to prepare library for sequencing with Revio +SPRQ/SPRQ-Nx or Vega Or Proceed to SMRT Link Sample Setup for preparing samples for Sequel II/IIe systems. **SAFE STOPPING POINT - Store at 4°C for up to 2 weeks and -20°C for long term**

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9. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 µL) for sequencing on the Vega system or Revio (+SPRQ/SPRQ-Nx). If samples are pooled prior to ABC or a custom volume is required, see Appendix A1. **The Polymerase kit used will depend on which sequencer or chemistry is being used (see below).**

Kit	PN
Revio SPRQ polymerase kit	103-496-900
Vega polymerase kit	103-426-500

✔	Step	Instructions
	9.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	25 µL

	9.2	Pipette-mix the **Annealing mix** and quick spin to collect liquid.
	9.3	Add **25 µL** of the **Annealing mix** to each library. Total volume should equal **50 µL.**
	9.4	Pipette-mix each sample and quick spin to collect liquid.
	9.5	Incubate at room temperature for **15 minutes**.
	9.6	During primer incubation, prepare the polymerase dilution (see below) and store on ice.
	9.7	To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase Dilution

✔	Tube	Component	Volume
	Yellow	Polymerase buffer	47 µL
	Purple	Sequencing polymerase	3 µL
		Total volume	**50 µL**

	9.8	Pipette mix the **polymerase dilution** and quick-spin to collect liquid.
	9.9	Add **50 µL of polymerase dilution** to primer annealed sample. Total volume should equal **100 µL**.
	9.10	Pipette-mix each sample and quick-spin to collect liquid.
	9.11	Incubate at **room temperature for 15 minutes.**
	9.12	Proceed immediately to the next step of the protocol to remove excess polymerase.

Post-binding cleanup with 1.3X SMRTbell cleanup beads

	9.13	Add **130 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample

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9.14	Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
9.15	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads
9.16	Place sample on an appropriate magnet and allow beads to separate fully from the solution
9.17	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH.** Proceed immediately to the elution. It is important not to let the beads dry out.
9.18	Remove sample from the magnet and **immediately** add **Loading buffer** to each tube and resuspend the beads by pipette mixing.

	Revio SPRQ Polymerase kit	Vega Polymerase kit
Loading buffer	25 µL	50 µL

9.19	Quick-spin the samples to collect any liquid from the sides of the tube.
9.20	Incubate at **room temperature** for 1**5 minutes** to elute DNA
9.21	Place sample on magnet and allow beads to separate fully from the solution.
9.22	Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a **new tube**. Discard the old tube with beads
9.23	Use **1 µL** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. **Important:** The **Qubit Flex** instrument is not compatible with measuring polymerase-bound library in Loading Buffer. Concentration readings will not be accurate.
9.24	Proceed to the **Loading Calculator** in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to Sequencing reagent plate. The recommended loading concentration is 200–300 pM.

PROTOCOL COMPLETE

Important: Polymerase-bound libraries can be stored at 4°C for up to 1 month, or at -20°C for up to 6 months prior to sequencing. Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles. Note that the Loading buffer is light sensitive.

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Appendix

A1. Annealing, binding, and cleanup (ABC) for custom volumes

This step is for preparing libraries for sequencing on PacBio HiFi sequencers. **Libraries or pools of libraries must be at a concentration of <20 ng/µL.** The sequencing polymerase is stable once bound to the HiFi library and can be stored at 4°C for 1 month or at -20°C for at least 6 months. Use the calculations below to determine reagent volumes based on input sample volume:

	SMRTbell library	Annealing buffer	Standard sequencing primer	Polymerase dilution
Volume (µL)	x	x/2	x/2	x*2
Example	100	50	50	200

See Section 9 - Annealing, binding, and cleanup (ABC) for full protocol.

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
Updated for SPRQ chemistry and the Vega system	04	February 2025
Updated for Revio SPRQ-Nx	05	May 2026

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