Generating PureTarget™ libraries with PureTarget kit 24 – manual protocol

Procedure & checklist

Before you begin

This procedure describes the workflow for manually generating PureTarget libraries.

Overview	
Samples	8–48 (processed in batches of 8)
Library prep time	8 hours +/- 2 hours for up to 24 samples
DNA input	
Quantity	1 – 4 µg in Elution buffer, TE buffer (pH 8.0, 0.1mM EDTA), or nuclease-free water
DNA size distribution	50% ≥30 kb on Femto Pulse system; DIN >8 on TapeStation system
Sample multiplexing	
Vega™ system	Up to 48 samples
Revio® system with SPRQ	Up to 48 samples

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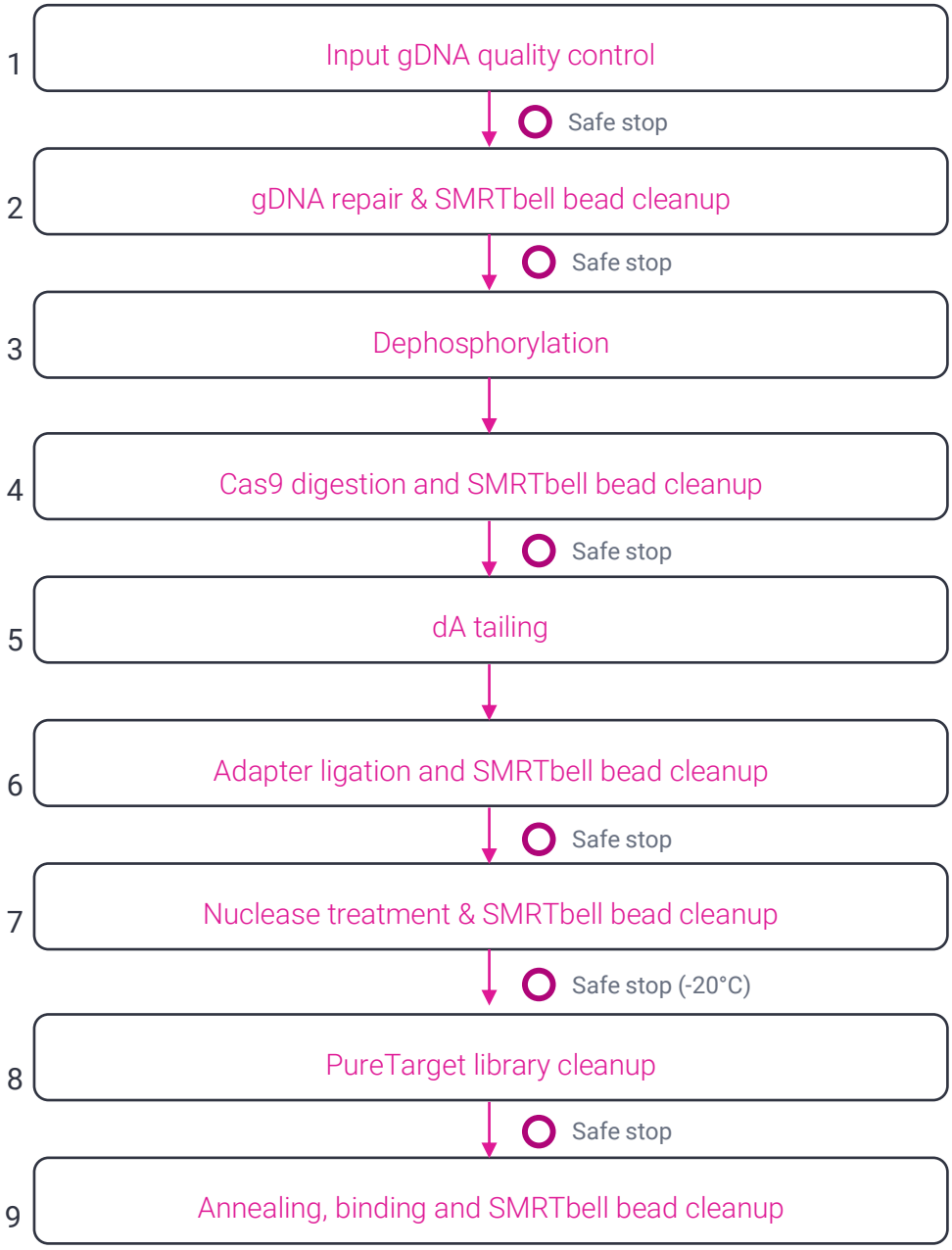
Workflow overview



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Materials and equipment

Recommended DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
TapeStation system	Agilent Technologies, Inc. G2992AA or G2991BA
DNA quantitation	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
Target enrichment and library preparation	
PureTarget™ repeat expansion panel 2.0 *or* PureTarget™ carrier panel *or* PureTarget™ control panel	PacBio® 103-633-100 PacBio® 103-633-200 PacBio® 103-633-300
PureTarget™ cleanup beads kit	PacBio® 103-633-000
PureTarget™ cleanup buffer kit	PacBio® 103-682-800
PureTarget™ kit 2.0	PacBio® 103-632-900*
SMRTbell® prep kit 3.0	PacBio® 102-141-700*
SMRTbell® cleanup beads-10mL	PacBio® 102-158-300*
Elution buffer	PacBio® 101-633-500*
SMRTbell® adapter index plate 96A (for barcoding) or SMRTbell® adapter index plate 96B (for barcoding) or SMRTbell® adapter index plate 96C (for barcoding) or SMRTbell® adapter index plate 96D (for barcoding)	PacBio® 102-009-200 PacBio® 102-547-800 PacBio® 102-547-900 PacBio® 102-548-000
Revio® SPRQ™ polymerase kit	PacBio® 103-496-900
Vega™ polymerase kit	PacBio® 103-426-500
General lab supplies and equipment	
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (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	Any MLS
Magnetic separation rack compatible with 1.5 mL tubes	Any MLS (e.g., Invitrogen DynaMag-2 Magnet 12321D)
Thermocycler compatible with 0.2 mL tube strips	Any MLS
Nuclease-free (NF) water	Any MLS, molecular biology grade
1.5 mL DNA LoBind tubes	Eppendorf 022431021

*Sold together as part of the PureTarget™ kit 24 bundle

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General best practices

DNA input

For optimal performance, this protocol recommends high-quality, high molecular weight (HMW) human gDNA with at least 50% of the mass of DNA in molecules ≥30 kb in length, corresponding to a genome quality number (GQN) of ≥5 at 30 kb based on the Agilent Femto Pulse system, or a DIN value >8 on the Agilent TapeStation. For low-quality human gDNA, it is still feasible to proceed, but lower on-target coverage is expected.

Note: Removal of RNA with RNAse is required for any DNA extraction method used. Failure to remove RNA may result in sequencing inhibition.

The supported sample types are:

- **Human blood genomic DNA**: extracted by Nanobind® RBC lysis PanDNA kit, Nanobind Whole blood manual CBB kit/PanDNA kit, Nanobind HT 200 µL protocol, Nanobind HT 1 mL protocol.
- **Human B-lymphocyte cell lines**: extracted by Nanobind CBB, Nanobind PanDNA, Nanobind CBB HT; Coriell cell line gDNA (GQN_{30kb} ≥5).
- **Human saliva DNA**: extracted by Nanobind® PanDNA, Nanobind® CBB HT. A diluted AMPure PB bead cleanup is required to proceed with this sample type. Please refer to Appendix A1 for details.

  Important: To ensure that there is sufficient mass for the gDNA repair step, 3 µg of Nanobind saliva gDNA is the recommended input mass for 3.1x dilute (35%) AMPure. Recoveries range from 50–80%. Less than 50% recovery gDNA from 3.1x dilute AMPure PB may indicate a low-quality sample, and low coverage may be observed. It is recommended to start at the gDNA repair step with 2 µg per sample, with a maximum of 24-plex on Revio + SPRQ or Vega for the best performance. Note that samples with a higher bacterial content will have lower target coverage. Bacterial contamination will vary from sample to sample.

For information on the performance of other extraction methods, see Application note - Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing.

Overall, the recommended mass of DNA is **2 µg per sample** to ensure that there are sufficient gene copies to load and maximize sequencing coverage. This protocol is compatible with 1–4 µg per sample. The recommended maximum total DNA mass per SMRT cell across all multiplexed samples is outlined in the following table. For example, a multiplex of 48 samples on Revio (+SPRQ) should not exceed 1ug per sample on average.

System	Min gDNA input	Max gDNA input
Vega	16 µg	100 µg
Revio (+SPRQ)	16 µg	50 µg

Multiplexing samples

Vega system

Up to 48 samples can be barcoded and sequenced per Vega SMRT Cell. These samples should be processed in batches of 8, 16, 24, 32, 40, or 48 samples.

Revio system with SPRQ chemistry

Up to 48 samples can be barcoded and sequenced per SMRT cell on the Revio system with SPRQ chemistry. These samples should be processed in batches of 8, 16, 24, 32, 40, or 48 samples.

Note: Each PureTarget kit supports the preparation of 24 samples. Therefore, two library prep kits are required to generate a Revio/Vega run of 32, 40, or 48 samples.

Reagent and sample handling

- Room temperature is defined as any temperature in the range of 18–23°C for this protocol.
- Do not vortex enzymes.
- Bring SMRTbell cleanup beads to room temperature. Always vortex immediately prior to use.
- Bring Qubit reagents to room temperature prior to use.
- Thaw frozen reagents at room temperature. Place on ice after thawing.
- Keep master mixes involving temperature-sensitive reagents on ice until use.
- Quick-spin all reagents in a microcentrifuge to collect liquid at the bottom prior to use.
- Samples can be stored at the specified temperature at the safe stopping points listed in the protocol.

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

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Library preparation reagents

Kits stored at -20°C.

Step used	Tube color	Reagent
PureTarget kit 2.0		
Dephosphorylation	Blue	Phosphatase
Cas9 digestion	Green	Cas9 Nuclease
Dephosphorylation/Cas9 digestion	Red	Cas9 buffer
dA tailing	Orange	dA tail buffer
dA tailing	Violet	*Taq* DNA Polymerase
dA tailing	Yellow	dATP
Nuclease treatment	White	Pure Target Nuclease
PureTarget panel		
Cas9 digestion	White	Repeat expansion panel 2.0 *or*
	Purple	Carrier panel *or*
	Orange	Control panel
SMRTbell prep kit 3.0		
gDNA repair	Purple	Repair buffer
gDNA repair	Blue	End repair mix
gDNA repair	Green	DNA repair mix
Adapter ligation	Yellow	Ligation mix
Adapter ligation	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix
Nuclease treatment	Light purple	Nuclease buffer
SMRTbell adapter index plate		
Adapter ligation	White plate	SMRTbell adapter index plate 96A, 96B, 96C, or 96D

PureTarget cleanup beads kit

Keep at room temperature.

Component	Tube color
PureTarget cleanup wash buffer*	Bottle, white
PureTarget cleanup binding buffer	Bottle, white
PureTarget cleanup beads	Clear

Important: *Prior to the 1st use of the PureTarget cleanup beads kit, add 15mL of 200 Proof ethanol to PureTarget cleanup wash buffer and mix well. Ensure bottle cap is tightly screwed on and store bottles upright to prevent leakage.

PureTarget cleanup buffer Kit

Keep at room temperature.

Component	Tube color
PureTarget cleanup buffer 1	Red
PureTarget cleanup buffer 2	Blue
PureTarget cleanup buffer 3	Green

Polymerase kit

Note: Bring the Loading buffer to room temperature prior to use. The Loading buffer is light sensitive and should be protected from light when not in use.

Kit stored at -20°C.

Component	Tube color
Annealing buffer	Light blue
Standard sequencing primer	Light green
Polymerase buffer	Yellow
Loading buffer	Green
Dilution buffer	Blue
Sequencing polymerase	Purple
Sequencing control	Red

Note: The Revio SPRQ polymerase kit supplies reagent volumes sufficient for 12 SMRT Cells for PureTarget. The Vega polymerase kit supplies reagent volumes sufficient for 4 SMRT Cells for PureTarget.

Thermocycler conditions

Program thermocycler(s) prior to beginning the protocol for the first time. If the lid temperature is not adjustable, set the lid temperature to "ON".

Note: Maintaining the lid temperature at 10°C higher than the incubation temperature is designed to minimize condensation. If the lid does not cool to the recommended values, performance will not be impacted. As long as the block temperature is accurate and the lid temperature is at least 10°C above the incubation temperature for heated steps, the enzymatic reactions will proceed as expected.

1.DNA repair program

Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37°C
2	30 min	52°C
3	Hold	4°C

2.Dephosphorylation program

Set the lid temperature to ≥95°C.

Step	Time	Temperature
1	20 min	37°C
2	5 min	85°C
3	Hold	4°C

3. Cas9 digestion program

Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

4. dA-tail program

Set the lid temperature to ≥95°C.

Step	Time	Temperature
1	1 min	37°C
2	5 min	72°C
3	Hold	4°C

5. Adapter ligation program

Set the lid temperature to “OFF” or ≥30°C.

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

6. Nuclease treatment program

Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

Workflow steps

1. Input DNA quality control and dilution

Before you begin, evaluate the size distribution of the input DNA using an Agilent Femto Pulse system or TapeStation system to determine whether it is suitable for the protocol.

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to **room temperature**.
	1.2	Pulse vortex and/or pipette-mix each sample 5 times to homogenize the DNA in solution. **Note:** For viscous input DNA, it is important to homogenize the extracted DNA prior to start of the protocol. To homogenize the DNA, pulse-vortex and/or pipette-mix full sample volume with standard (not wide bore) tips. These steps will maintain HMW of your DNA but will improve accuracy of quantification and subsequent handling.
	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	Measure DNA size distribution with a Femto Pulse system using the **gDNA 165 kb analysis kit.** **Or** Measure DNA size distribution with a **TapeStation system** using the Genomic DNA ScreenTape Analysis
	1.7	Aliquot or bring **1- 4 µg DNA** to a final volume of **43 µL per sample** (24- 93 ng/µL) with nuclease-free water or Elution buffer.

SAFE STOPPING POINT - Store at 4°C

2. gDNA repair

This step repairs input genomic DNA, which increases target coverage when using lower quality gDNA.

Important: For **Nanobind extracted blood** (whole blood or RBC lysis method) gDNA, this step can be skipped. For other sample types or extraction methods, the gDNA repair step is required to ensure sufficient coverage.

Important: For **Nanobind extracted saliva** gDNA, the 3.1x 35% AMPure PB cleanup is required before the start of the library prep, please refer to Appendix A1 for details.

✔	Step	Instructions
	2.1	Add the following components to a microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below.

gDNA repair master mix

✔	Reagent	Component	Volume per sample	8 reactions*	16 reactions*	24 reactions*
	Purple	Repair buffer	5 µL	48 µL	96 µL	144 µL
	Green	DNA repair mix	1.8 µL	17.3 µL	34.6 µL	51.8 µL
	Blue	End repair mix	0.2 µL	1.9 µL	3.8 µL	5.8 µL
		Total volume	**7µL**	**67.2 µL**	**134.4 µL**	**201.6 µL**

*20% overage included

	2.2	Pipette-mix and quick-spin the gDNA repair master mix.
	2.3	Add **7 µL** of the **gDNA repair master mix** to each gDNA sample from step 1.7 for a total reaction volume of **50 µL**.
	2.4	Pipette-mix and quick-spin.
	2.5	Run the DNA repair thermocycler program. Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37°C
2	30 min	52°C
3	Hold	4°C

1.2X SMRTbell bead cleanup

	2.6	Add **60 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	2.7	Pipette-mix the beads until evenly distributed. **Bead clumping may occur and is not a concern. Avoid over-pipetting as it may cause DNA/bead mixture to stick to the pipette tip.**
	2.9	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	2.10	Place on a magnetic separation until beads separate fully from the solution.

2.11	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
2.12	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each well. After **30 seconds**, remove the 80% ethanol and discard.
2.13	Repeat the previous step.
2.14	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
2.15	Remove sample from the magnetic rack. **Immediately** add **68 µL** of **Elution buffer** to each well and resuspend the beads.
2.17	Incubate at **room temperature** for **5 minutes** to elute DNA.
2.18	Place on a magnetic separation rack until beads separate fully from the solution.
2.19	Slowly transfer **68 µL** cleared eluate without disturbing the beads to a new tube strip. Discard the old tube strip with beads.
2.20	Optional QC step: Take a 1 µL each sample and measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit. **Expect a step recovery of 70-90% per sample.**
2.21	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

3. Dephosphorylation

This step dephosphorylates the genomic DNA 5' termini, which prevents adapter ligation to non-targeted genomic DNA.

✔	Step	Instructions
	3.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below.

Dephosphorylation master mix

✔	Tube color	Component	Volume per sample	8 reactions*	16 reactions*	24 reactions*
	Red	Cas9 buffer	8 µL	76.8 µL	153.6 µL	230.4 µL
	Blue	Phosphatase	5 µL	48 µL	96 µL	144 µL
		Total volume	**13 µL**	**124.8 µL**	**249.6 µL**	**374.4 µL**

*20% overage included

3.2	Pipette-mix the **Dephosphorylation master mix** and quick-spin in a microcentrifuge to collect liquid.

3.3	Add **13 µL** of the **Dephosphorylation master mix** to each sample from step 2.19 for total reaction volume of **80 µL**.
3.4	Pipette-mix each sample.
3.5	Run the **Dephosphorylation** thermocycler program. Set the lid temperature to ≥95°C.

Step	Time	Temperature
1	20 min	37°C
2	5 min	85°C
3	Hold	4°C

3.6	Proceed to the next step of the protocol.

4. Cas9 digestion and SMRTbell cleanup

This step enables digestion of double-stranded DNA at targeted regions mediated by Cas9-gRNA ribonucleoprotein complex formation.

✔	Step	Instructions
	4.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below.

Cas9 digestion master mix

✔	Reagent	Component	Volume per sample	8 reactions*	16 reactions*	24 reactions*
		Nuclease free water	2 µL	19.2 µL	38.4 µL	57.6 µL
	Red	Cas9 buffer	0.5 µL	4.8 µL	9.6 µL	14.4 µL
	Green	Cas9 Nuclease	0.5 µL	4.8 µL	9.6 µL	14.4 µL
	variable	PureTarget panel**	2 µL	19.2 µL	38.4 µL	57.6 µL
		Total volume	**5.0 µL**	**48 µL**	**96 µL**	**144 µL**

*20% overage included

**Repeat expansion 2.0, Carrier, or Control panel with custom guide RNA

4.2	Pipette-mix **Cas9 digestion master mix** and quick-spin in a microcentrifuge to collect liquid.
4.3	Add **5 µL** of the Cas9 digestion master mix to each sample for total reaction volume of **85 µL.**
4.4	Pipette-mix each sample

4.5 Run the **Cas-9 digestion** thermocycler program. Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

1.2X SMRTbell bead cleanup

4.7	Add **102 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
4.8	Pipette-mix the beads slowly until evenly distributed. **Bead clumping may occur and is not a concern. Avoid over-pipetting as it may cause DNA/bead mixture to stick to the pipette tip.**
4.10	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
4.11	Place on a magnetic separation rack until beads separate fully from the solution.
4.12	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
4.13	Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.
4.14	Repeat the previous step.
4.15	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
4.16	Remove sample from the magnetic rack. **Immediately** add **41 µL** of Elution buffer to each well and resuspend the beads.
4.18	Incubate at **room temperature** for **5 minutes** to elute DNA.
4.19	Place on a magnetic separation rack until beads separate fully from the solution.
4.20	Slowly transfer **41 µL** cleared eluate without disturbing the beads to a **new tube strip**. Discard old tube strip with beads.
4.21	Optional QC step: Take a 1 µL aliquot from each sample and dilute with 9 µL of Elution buffer or water. Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit. **Note: Expect a step recovery of 50-80% per sample.**
4.22	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

5. dA-tail

This step enables A-tailing of DNA 3’ ends after Cas9-gRNA digestion at targeted regions.

✔	Step	Instructions
	5.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below.

dA-tailing master mix

✔	Reagent	Component	Volume per sample	8 reactions*	16 reactions*	24 reactions*
		Nuclease free water	3.5	33.6 µL	67.2 µL	100.8 µL
	Orange	dA tail buffer	5 µL	48 µL	96 µL	144 µL
	Yellow	dATP (100 mM)	0.2 µL	1.9 µL	3.8 µL	5.8 µL
	Violet	*Taq* DNA polymerase	1.3 µL	12.5 µL	25 µL	37.4 µL
		Total volume	**10 µL**	**96 µL**	**192 µL**	**288 µL**

*20% overage included

	5.2	Pipette-mix the **dA-tailing master mix** and quick-spin in a microcentrifuge to collect liquid.
	5.3	Add **10 µL** of the **dA-tailing master mix** to each sample for a total reaction volume of **50 µL**.
	5.4	Pipette-mix each sample.
	5.5	Run the **dA-tail** thermocycler program. Set the lid temperature to ≥95°C.

Step	Time	Temperature
1	1 min	37°C
2	5 min	72°C
3	Hold	4°C

	5.6	Proceed to the next step of the protocol.

6. Adapter ligation and SMRTbell cleanup

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

✔	Step	Instructions
	6.1	Add **4 µL SMRTbell adapter index** to each sample from Section 5 and pipette mix.
	6.2	Add the following to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below. **Note:** Aspirate and dispense viscous ligation mix slowly to reduce liquid sticking to the exterior and interior wall of the pipette tip.

Ligation master mix

✔	Reagent	Component	Volume per sample	8 reactions*	16 reactions*	24 reactions*
	Yellow	Ligation mix	30 µL	264 µL	528 µL	792 µL
	Red	Ligation enhancer	1 µL	8.8 µL	17.6 µL	26.4 µL
		Total volume	**31 µL**	**272.8 µL**	**545.6 µL**	**818.4 µL**

*10% overage included

6.3	Pulse-vortex mix the **Ligation master mix** and quick-spin in a microcentrifuge to collect liquid.
6.4	Add **31 µL** of **Ligation master mix** to each sample from previous step for a total volume of **85 µL.**
6.5	Pipette-mix each sample
6.6	Run the Adapter ligation thermocycler program. Heating of the lid is not necessary.

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

Cleanup with 1X SMRTbell cleanup beads

6.7	Add **85 µL** (1X) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
6.8	Pipette-mix until evenly distributed.
6.9	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
6.10	Place on a magnetic rack until beads separate fully from the solution.
6.11	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
6.12	Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.
6.13	Repeat the previous step.
6.14	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid.

	• Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
6.15	Remove from the magnetic rack. **Immediately** add **41 µL** of **Elution buffer** to each sample and resuspend the beads.
6.16	Incubate at **room temperature** for **5 minutes** to elute DNA.
6.17	Place in a magnetic separation rack until beads separate fully from the solution.
6.18	Slowly transfer **41 µL** cleared eluate without disturbing the beads to new strip tubes.
6.19	Optional QC step: Take a **1 µL** from each sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** **Expect a step recovery of 70-90% per sample.**
6.20	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

7. Nuclease treatment

This step removes DNA fragments that have not formed SMRTbell libraries.

✔	Step	Instructions
	7.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus overage according to the table below.

Nuclease master mix

✔	Reagent	Component	Vol per sample	8 reactions*	16 reactions*	24 reactions*
	Light purple	Nuclease buffer	5 µL	44 µL	88 µL	132 µL
	Light Green	Nuclease mix	5 µL	44 µL	88 µL	132 µL
	White	PureTarget Nuclease**	0.5 µL	4.4 µL	8.8 µL	13.2 µL
		Total volume	**10.5 µL**	**92.4 µL**	**184.8 µL**	**277.2 µL**

*10% overage included

**Located in the PureTarget kit 2.0

7.2	Pipette-mix the **Nuclease master mix** and quick-spin in a microcentrifuge to collect liquid.
7.3	Add **10.5 µL** of **Nuclease master mix** to **40 µL** of each sample for a total volume of **50.5 µL**.
7.4	Pipette-mix each sample.

7.5 Run the **Nuclease treatment** thermocycler program. Set the lid temperature to ≥65°C.

Step	Time	Temperature
1	60 min	37° C
2	Hold	4° C

Sample pooling and 1.2X SMRTbell cleanup

7.6	In a 1.5 mL DNA LoBind tube combine nuclease treated libraries from step 7.5 in groups of 8 **(8 x 50.5 µL)** for a final sample volume of **404 µL.**
7.7	Add **485 µL** of SMRTbell cleanup beads to each pooled nuclease-treated sample. Pipette-mix until the beads are evenly distributed.
7.8	Quick-spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
7.9	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
7.10	Place the tube in a magnetic separation rack until beads separate fully from the solution.
7.11	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
7.12	Slowly dispense **1 mL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.
7.13	Repeat the previous step.
7.14	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
7.15	Remove from the magnetic rack. **Immediately** add **55 µL** of **Elution buffer** to each tube and resuspend the beads by pipette mixing until resuspended.
7.16	Incubate at **room temperature** for **5 minutes** to elute DNA.
7.17	Place on a magnetic separation rack until beads separate fully from the solution.
7.18	Slowly transfer **55 µL** cleared eluate from each nuclease reaction and **pool into a new 1.5 mL DNA LoBind tube.**

Plex	Total pooled volume
8-plex	55 µL
16-plex	110 µL*
24-plex	165 µL*
48-plex	330 µL*

* requires concentration step if volume is >55 µL – proceed to step 7.20

7.19 Proceed to the step 7.20 if the pooled volume is greater than **55 µL**; otherwise proceed to step 7.32 for QC.

Concentration of samples with a 1.2X SMRTbell cleanup

7.20 Add **1.2X volume** of SMRTbell cleanup beads to the sample from step **7.18**. For example, a 24-plex prep will have a sample of volume **165 µL**, which requires **198 µL** (**1.2X**) SMRTbell cleanup beads.

Plex	Total pooled volume	SMRTbell cleanup beads volume
16-plex	110 µL	132 µL
24-plex	165 µL	198 µL
48-plex	330 µL	396 µL

7.21	Pipette-mix until the beads are evenly distributed.
7.22	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
7.23	Place on a magnetic separation rack until beads separate fully from the solution.
7.24	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
7.25	Slowly dispense **1 mL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, remove the 80% ethanol and discard.
7.26	Repeat the previous step.
7.27	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
7.28	Remove the tube the magnetic rack. **Immediately** add **55 µL** of **Elution buffer** and pipette mix until fully resuspended.
7.29	Incubate at **room temperature** for **5 minutes** to elute DNA.
7.30	Place the tube in a magnetic separation rack until beads separate fully from the solution.
7.31	Slowly transfer **55 µL** cleared supernatant without disturbing the beads to a **new 1.5 mL DNA LoBind tube**.
7.32	QC step: Take a **1 µL** aliquot from each sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** **Expect an overall recovery of 0.05-0.3% relative to the starting gDNA total mass. For example, starting with 1 µg input per sample, the final mass of a pooled 48-plex library recovered at this step is expected to be in the range of 25 ng–150 ng. A higher than expected library yield may indicate inefficient nuclease digestion, or sample incompatibility with the protocol. Refer to Appendix A2 section for recommendations to further digest the sample.**
7.33	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at -20°C

8. PureTarget cleanup of the SMRTbell library

This step will further clean the library for sequencing. Due to the low mass of DNA library, it's strongly recommended to use DNA LoBind tubes for this step and onward.

Perform one cleanup per final pool of 8-48 samples.

Important: Prior to the 1st use of the PureTarget cleanup beads kit, add 15mL of 200 Proof ethanol to PureTarget cleanup wash buffer and mix well.

✔	Step	Instructions
	8.1	Add **28 µL PureTarget cleanup buffer 1** (🔴) to **55 µL SMRTbell library** from step 7.31 and pipette-mix.
	8.2	Add the following components to a new microcentrifuge tube. **PureTarget cleanup buffer 2+3 mix** ✔ Reagent: Blue — Component: PureTarget cleanup buffer 2 — Volume per sample: 14 µL — Volume (overage included): 18 µL ✔ Reagent: Green — Component: PureTarget cleanup buffer 3 — Volume per sample: 14 µL — Volume (overage included): 18 µL **Total volume** — **28 µL** — **36 µL** Pulse vortex to mix and quick-spin. **Important: PureTarget cleanup buffer 2 and buffer 3 must be pre-mixed prior to adding to sample. If added individually to sample, sample recovery may be low.**
	8.3	Add **28 µL PureTarget cleanup buffer 2+3 mix** to the mixture from step 8.1 and pipette-mix. The solution will turn opaque and cloudy after homogenous mixing. Incubate at room temperature for 1 minute.
	8.4	Centrifuge at 13,000 rcf for 2 minutes at room temperature.
	8.5	Taking care to avoid the pellet, transfer **95 µL** of the clear supernatant to a clean 1.5 mL LoBind tube. Discard the tube with pellet.
	8.6	Thoroughly resuspend **PureTarget cleanup beads** by pulse-vortexing and quick spin. Add **5 µL** of fully resuspended PureTarget cleanup beads to **95 µL** of the sample from step 8.5.
	8.7	Add **100 µL** of **PureTarget cleanup binding buffer** to the sample from step 8.6.
	8.8	Pipette-mix until evenly distributed. Incubate at room temperature for **15 mins**, with **periodic pipette-mixing to ensure that beads are resuspended during the entire incubation**.
	8.9	Quick-spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
	8.10	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	8.11	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	8.12	Slowly dispense **500 µL** of **PureTarget cleanup wash buffer (with ethanol added)** into the tube. Remove the tube from the magnetic separation rack and gently resuspend the beads. Close the tube cap and invert the tube 5 times to wash off any residual PureTarget Cleanup Binding Buffer.

8.13	Place the tube in a magnetic separation rack until beads separate fully from the solution.
8.14	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
8.15	Slowly dispense **500 µL** of **fresh 80% ethanol** into the tube **without** disturbing the beads.
8.16	After **30 seconds**, remove the 80% ethanol and discard.
8.17	It is critical to remove residual 80% ethanol for efficient elution: • 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. • Air dry the bead pellet for 2 mins.
8.18	Remove the tube from the magnetic rack. Add **26 µL** of **Elution buffer** and resuspend the beads by gentle vortexing.
8.19	Incubate at **room temperature** for **15 minutes** to elute DNA, with gentle vortexing every 5 mins to fully resuspend the beads.
8.20	Place the tube in a magnetic separation rack until beads separate fully from the solution.
8.21	Slowly transfer **26 µL** cleared supernatant without disturbing the beads to a new **1.5 mL DNA LoBind tube.**
8.22	QC step: Take a **1 µL** aliquot from the sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.** **Expect a step recovery of 40-60%, and an overall recovery of 0.02-0.2% relative to starting gDNA total mass.** For example, starting with 1.0 µg gDNA per sample for a 48-plex prep to load on Revio with SPRQ, the final mass of the pooled 48-plex library recovered at this step is expected to be in the range of 10 ng– 100 ng. SAFE STOPPING POINT – Store at 4°C overnight or -20°C long term

9. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 µL) for sequencing on supported platforms.

✔	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 for a total volume of **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	44 µL
	Purple	Sequencing polymerase	6 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 for a total volume of **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.

1.2X SMRTbell bead cleanup

	9.13	Add **120 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample
	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	23 µL	98 µL

9.19	Quick-spin the samples to collect any liquid from the sides of the tube.
9.20	Incubate at **room temperature** for **15 minutes** to elute DNA
9.21	Place sample on magnet and allow beads to separate fully from the solution.
9.22	Slowly transfer the cleared eluate without disturbing the beads to a **new tube protected from light**. Discard the old tube with beads. The sample is now ready for sequencing (see Section 10).

PROTOCOL COMPLETE

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

10. Preparing sequencing internal control and sample for sequencing

Step	Instructions
10.1	Dilute the sequencing control using the volumes listed below.

Dilution 1	
Component	Volume
Dilution buffer	19 µL
Sequencing control	1 µL
Total volume	20 µL

Pipette mix and quick-spin in a microcentrifuge to collect liquid.

10.2 Dilute Dilution 1 using the volumes listed below.

Dilution 2	
Component	Volume
Dilution buffer	19 µL
Dilution 1	1 µL
Total volume	20 µL

Pipette mix and quick-spin in a microcentrifuge to collect liquid.

10.3 Dilute Dilution 2 using the volumes listed below.

Dilution 3	
Component	Volume
Dilution buffer	19 µL
Dilution 2	1 µL
Total volume	20 µL

Pipette mix and quick-spin in a microcentrifuge to collect liquid.

10.4 Prepare sample for sequencing. Add the following components for the appropriate sequencing platform.

Sequencing sample and control		
Component	Revio SPRQ	Vega
Sample from step 9.22	23 µL	98 µL
Diluted internal sequencing control (Dilution 3)	1 µL	3 µL
Total volume	24 µL	101 µL

10.5 Load the Sequencing sample and control prepared in step 10.4. Load the exact volume indicated for the appropriate sequencing platform.

Revio SPRQ: 23 µL
Vega: 100 µL

10.6 For Run Design, ensure the following specifications are selected:
Use Adaptive Loading: *Off*
Application: *PureTarget repeat expansion/carrier/custom*
Analysis Workflow:

- PureTarget repeat expansion: *PureTarget repeat expansion*
- PureTarget carrier: *Target Enrichment*
- PureTarget custom: *Target Enrichment*

Target and repeat definitions:

- PureTarget repeat expansion: *PureTarget repeat expansion panel 2.0*

- PureTarget carrier: *PureTarget carrier panel 1.0*
- PureTarget custom: user-provided bed file. Please reference the SMRT Link User Guide for bed file format specifications

If setting up Target Enrichment analysis for PureTarget custom or in SMRT Analysis after sequencing, the user must select the following specifications under "Advanced":

- Include Fail Reads: On
- Padding around regions (bp): 0

Appendix

A1. Diluted AMPure® PB bead cleanup and size selection

The AMPure PB bead (PN 100-265-900) size selection step will deplete fragments shorter than 3 kb and may remove contaminants that inhibit library preparation. This step is required for saliva DNA to yield the best performance.

Important: To ensure that there is sufficient mass for the gDNA repair step, 3 µg of Nanobind saliva gDNA is the recommended input mass for 3.1x dilute (35%) AMPure PB. Recoveries range from 50–80%. Less than 50% recovery gDNA from 3.1x dilute AMPure PB may indicate a low-quality sample, and low coverage may be observed. It is recommended to start at the gDNA repair step with 2 µg per sample, with a maximum of 24-plex on Revio + SPRQ or Vega system for the best performance. Note that samples with a higher bacterial content will have lower target coverage. Bacterial contamination will vary from sample to sample.

Recovery is sensitive to bead concentrations; therefore, ensure accurate pipetting volumes when diluting AMPure PB beads.

✔	Step	Instructions for AMPure PB bead size selection
	A1.1	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. **The AMPure PB dilution may be scaled as appropriate for number of samples.**
	A1.2	Prepare extracted saliva gDNA in 45uL with Elution buffer or nuclease-free water. The starting mass for this cleanup can range from **1.5 – 6 µg, but >3 µg is recommended**. Add **3.1X v/v** (140 µL) of resuspended, room-temperature **35% AMPure PB beads** to 45 µL of gDNA sample.
	A1.3	Pipette-mix the beads until evenly distributed.
	A1.4	Incubate at **room temperature** for **20 minutes** to allow DNA to bind beads.
	A1.5	Place sample on an appropriate magnet and allow beads separate fully from the solution.
	A1.6	Slowly remove the cleared supernatant without disturbing the beads.
	A1.7	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into each sample. After **30 seconds**, pipette off the 80% ethanol and discard.

A1.8	Repeat the previous step.
A1.9	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid. • Place samples back on the magnet and wait until beads separate fully from the solution. • Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.
A1.10	Remove samples from the magnet and **immediately** add **44 µL** of **elution buffer** to each sample.
A1.11	Pipette-mix the beads until evenly distributed.
A1.12	Incubate at **room temperature** for **5 minutes** to elute DNA of the beads.
A1.13	Place samples on the magnet and allow the beads to separate fully from the solution.
A1.14	Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube.
A1.15	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. The recovery should be 50–80% as measured from the input mass. For gDNA samples that have less than 40% cleanup recovery, it is recommended not to proceed as the size and quality of gDNA are likely not suitable. Optional: Further dilute each aliquot to **250 pg/µL** with Femto Pulse dilution buffer. Measure gDNA size distribution with a Femto Pulse system.
A1.16	The sample is now ready for gDNA repair, or store at 4°C overnight or -20°C long term

Appendix A1 COMPLETE

A2. Re-run nuclease digestion on high yield libraries

A library recovery of >0.3% after the nuclease step may indicate that the nuclease digestion is suboptimal or that samples contain impurities inhibiting nuclease digestion. To minimize impact on sequencing performance, an additional nuclease digestion on the pooled library (whether it's 8-plex or 48-plex) can be carried out after step 7.33 to further remove non-SMRTbell molecules. There are sufficient reagents to enable a second digestion of the pooled sample.

✔	Step	Instructions for Rerun nuclease digestion on the pooled library
	A2.1	To the **55 µL** library from step 7.33, add the following reagents for a final volume of 67 µL.

PureTarget nuclease re-digestion mix

✔	Reagent	Component	Volume per sample
	Light purple	Nuclease buffer	6.5 µL
	Light Green	Nuclease mix	5.0 µL
	White	PureTarget nuclease	0.5 µL
		Total volume	**12 µL**

	A2.2	Pipette-mix the sample. Quick-spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
	A2.3	Run the following **Nuclease treatment** thermocycler program with the lid set to ≥50°C.

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

Sample pooling and 1.2X SMRTbell cleanup

	A2.4	Add **81 µL** of SMRTbell cleanup beads to the nuclease re-treated sample. Pipette-mix until the beads are evenly distributed.
	A2.5	Quick-spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
	A2.6	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads.
	A2.7	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	A2.8	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	A2.9	Slowly dispense **200 µL** of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, remove the 80% ethanol and discard.
	A2.10	Repeat the previous step.
	A2.11	Remove residual 80% ethanol: • Remove the samples from the magnet and quick-spin to collect liquid.

- Place samples back on the magnet and wait until beads separate fully from the solution.
- Carefully pipette-off the residual 80% ethanol without disturbing the bead pellet and discard.

A2.12	Remove from the magnetic rack. **Immediately** add **56 µL** of **Elution buffer** and resuspend the beads by pipette mixing until resuspended.
A2.13	Incubate at **room temperature** for **5 minutes** to elute DNA.
A2.14	Place on a magnetic separation rack until beads separate fully from the solution.
A2.15	Slowly transfer **56 µL** cleared eluate **into a new 1.5 mL DNA LoBind tube. Proceed to step 8.1.** QC step: Take a **1 µL** aliquot from the sample and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit.**
A2.16	**If the initial nuclease digestion is not sufficient, a step recovery of 20-50% is expected, indicating a further removal of non-SMRTbell molecules.** **A step recovery of >70% indicates the initial nuclease digestion was effective, but a large mass of SMRTbells were formed in the library prep. This typically indicates the presence of a high mass of off-target SMRTbells, possibly due to incompatible extraction methods or sample types.**

Appendix A2 COMPLETE

Revision history (description)	Version	Date
Initial release	01	September 2025
Minor updates, updated DIN to >8, and updated guidance for saliva samples	02	October 2025
Included additional spin steps to remove residual ethanol and updated library complex storage condition	03	November 2025