Generating PureTarget™ repeat expansion panel libraries

Procedure & checklist

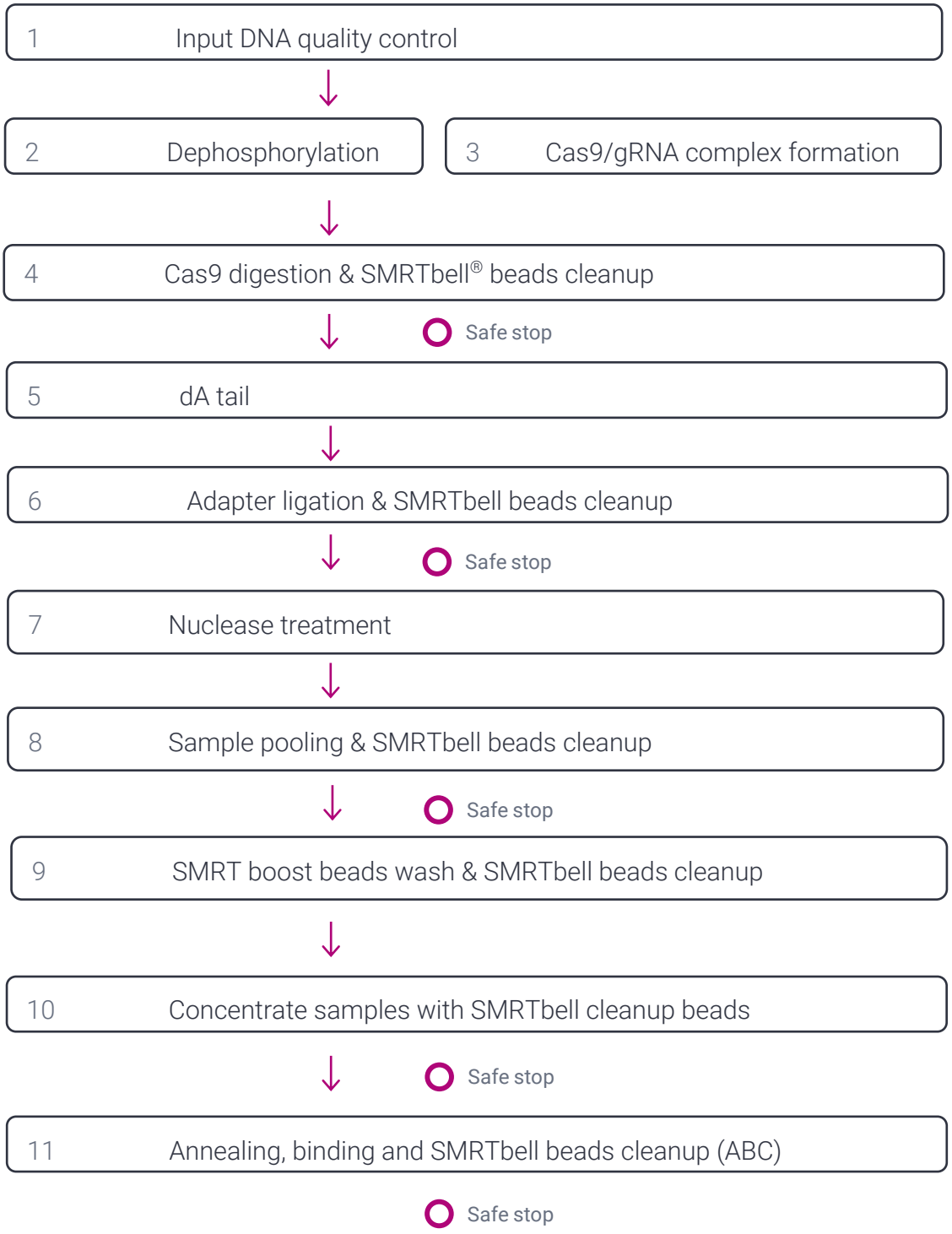
Before you begin

This procedure describes the workflow for generating PureTarget repeat expansion libraries.

Overview	
Samples	8–24 (processed in batches of 8)
Library prep time	8 hours +/- 2 hours for up to 24 samples
Annealing binding clean up time	1 hour +/- 10 minutes
DNA input	
Quantity	2 µg in Buffer EB, TE buffer (pH 8), or nuclease-free water
DNA size distribution	50% ≥30 kb
Sample multiplexing	
Sequel® II systems	Up to 24 samples
Revio™ system	Up to 48 samples

103-329-400 REV02 APR2024

Workflow overview



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Required materials and equipment

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
Target enrichment and library preparation	
PureTarget™ repeat expansion panel	PacBio® 103-390-400
PureTarget™ beads kit (store at 4C upon arrival) • SMRT boost beads • SMRT boost buffer	PacBio® 103-234-800*
PureTarget™ repeat expansion targeting kit (store at -20C upon arrival) • Cas9 Buffer • Phosphatase • Cas9 Nuclease • dA tail buffer • dATP (10mM) • *Taq* DNA polymerase • gRNA mix (5 µM)	PacBio® 103-234-700*
SMRTbell® prep kit 3.0 (store at -20C upon arrival) • Repair buffer • Ligation mix • Ligation enhancer • Nuclease buffer • Nuclease mix • *End repair mix (not used in this protocol)* • *DNA repair mix (not used in this protocol)*	PacBio® 102-141-700*
SMRTbell® Cleanup Beads	PacBio® 102-158-300*
Low TE buffer (pH 8.0)	PacBio® 102-178-400*
SMRTbell® adapter index plate 96A (for barcoding)	PacBio® 102-009-200
Revio™ polymerase kit (store at -20C upon arrival) • Annealing buffer	PacBio® 102-793-100

• Standard sequencing primer • *Kinnex™ sequencing primer (not used in this protocol)* • Polymerase buffer • Sequencing polymerase • Dilution buffer • Sequencing control • Loading buffer	
Sequel® II binding kit 3.2 (store at -20C upon arrival) • Sequel® II annealing buffer • Sequel® II primer 3.2 • *Kinnex™ sequencing primer (not used in this protocol)* • Sequel® II polymerase dilution buffer • Sequel® II DNA polymerase 2.2 • Sequel® II ABC buffer • Sequel® II DNA internal control complex 3.2 • Sequel® II loading buffer 3.2	PacBio® 102-194-100
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Mini-tube rotator	Any MLS (e.g., Fisher Scientific 05-450-127)
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 (e.g., V&P Scientific VP 772F4-1 8-strip or Permagen MSR812 24-strip)
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™ repeat expansion panel kit (103-390-400)

General best practices

DNA input

For optimal performance, this protocol requires high-quality, high molecular weight (HMW) human gDNA with at least 50% of the mass of DNA in molecules at ≥30 kb in length, or genome quality number (GQN) of ≥5 at 30 kb based on the Agilent Femto Pulse system.

The supported sample type is high-quality, HMW genomic DNA extracted with the Nanobind PanDNA kit (PacBio 103-260-000). For human whole blood we recommend the RBC lysis extraction method. For human cell lines Nanobind PanDNA or Nanobind® CBB kit (PacBio 102-301-900) is supported.

The recommended mass of DNA is **2 µg per sample** to ensure there are sufficient gene copies to load and maximize sequencing coverage. This protocol is suitable for 1–4 µg per sample. We recommend a maximum total DNA of 75 µg on the Sequel system and 100 µg on the Revio system across all multiplexed samples.

Multiplexing samples

Sequel II/ Sequel IIe systems

Up to 24 samples can be barcoded and sequenced per SMRT® Cell on the Sequel II and Sequel IIe systems. These samples should be processed in batches of 8, 16, or 24 samples.

Revio system

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

For Revio runs with 8, 16, or 24 samples, follow this protocol as is.

For Revio runs with 32, 40, or 48 samples, process two workflows in parallel: 1) 24 samples following this protocol and 2) an additional 8, 16, or 24 samples following this protocol.

Each PureTarget repeat expansion panel kit supports the preparation of 24 samples. Therefore, two library prep kits are required to generate a Revio 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.

Mix all reagents well prior to use. Vortex-mix all buffers prior to use. Do not vortex enzymes.

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

Thaw all temperature-sensitive reagents on ice and keep on ice prior to use. Keep master mixes involving temperature-sensitive reagents on ice until use.

Safety precautions

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

Temperature-sensitive reagents		
Step used	**Tube**	**Reagent**
Dephosphorylation	Blue	Phosphatase
Cas9 digestion	Green	Cas9 Nuclease
Cas9 digestion	Purple	gRNA mix
dA Tailing	Light Blue	*Taq* DNA Polymerase
dA Tailing	Yellow	dATP
Adapter ligation		SMRTbell adapter index plate 96A
Adapter ligation	Yellow	Ligation mix
Adapter ligation	Red	Ligation enhancer
Nuclease treatment	Light green	Nuclease mix

Bring SMRTbell cleanup beads, SMRT boost beads, SMRT boost buffer, and Qubit reagents to room temperature for 30 minutes prior to use.

Pipette-mix all reactions by pipetting up and down 10 times. Use full-volume pipette mixing of all reactions to ensure thorough mixing of all reaction components.

Samples can be stored at -20°C at all safe stopping points listed in the protocol.

Thermocycler conditions

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

1.Dephosphorylation program

Set the lid temperature to 95°C.

Step	Time	Temperature
1	10 min	37°C
2	3 min	80°C
3	Hold	4°C

2. Cas9 gRNA complex formation program

For this and the rest of thermocycler programs, set the lid temperature to 75°C.

Step	Time	Temperature
1	10 min	37°C
2	Hold	4°C

3. Cas9 digestion program

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

4. dA-tail program

Step	Time	Temperature
1	20 min	37°C
2	5 min	72°C
3	Hold	4°C

5. Adapter ligation program

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

6. Nuclease treatment program

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 quantity and size distribution of input DNA using Agilent Femto Pulse system to determine whether it is suitable for the protocol (we recommend HMW DNA with GQN of ≥5 at 30 kb).

✔	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. For viscous input DNA, it is important to homogenize the extracted DNA prior to start of the protocol. To homogenize the DNA, pulse-vortex 5 times and/or pipette-mix full sample volume 5 times, up and down 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	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 165 kb analysis kit.**
	1.8	Aliquot or bring 1–4 µg DNA to a final volume of 67 µL **per sample** with nuclease-free water and transfer to an 8 tube PCR strip.

SAFE STOPPING POINT - Store at 4°C

2. Dephosphorylation

This step enables dephosphorylation of genomic DNA 5’ and 3’ ends, which prevents subsequent adapter ligation to non-targeted genomic DNA ends.

✔	Step	Instructions
	2.1	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 15% overage according to the table below.

Reaction Mix 1 (RM1)

✔	Tube color	Component	Volume per sample	Volume per 8-plex (With 15% overage)	Volume per 16-plex (With 15% overage)	Volume per 24-plex (With 15% overage)
	Red	Cas9 buffer	8 µL	73.6 µL	147.2 µL	220.8 µL
	Blue	Phosphatase	5 µL	46 µL	92 µL	138 µL
		Total volume	**13 µL**	**119.6 µL**	**239.2 µL**	**358.8 µL**

2.2	Pipette-mix **RM1** up and down 10 times (do not vortex). Pipette-mix full volume.
2.3	Quick-spin **RM1** in a microcentrifuge to collect liquid.
2.4	Add **13 µL** of the **RM1** to each sample from step 1.8 for total reaction volume of **80 µL**.
2.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
2.6	Quick-spin in a microcentrifuge to collect liquid.
2.7	Run the **dephosphorylation** thermocycler program. Set the lid temperature to 95°C.

Step	Time	Temperature
1	10 min	37°C
2	3 min	80°C
3	Hold	4°C

2.8	Once the dephosphorylation program is complete, take out the sample and keep on ice until step 4. Meanwhile, proceed to the next step of the protocol, Step 3 below.

3. Cas9 gRNA complex formation

This step enables Cas9-gRNA ribonucleoprotein complex formation required for subsequent targeting and digestion of genomic DNA in Step 4.

✔	Step	Instructions
	3.1	Add the following components in the order and volume listed below to a new PCR tube. Adjust component volumes for the number of samples being prepared, plus 25% overage according to the table below.

Reaction Mix 2 (RM2)

✔	Reagent	Component	Volume per sample	Volume per 8-plex With 25% overage	Volume per 16-plex With 25% overage	Volume per 24-plex With 25% overage
		NF water	1.75 µL	17.5 µL	35 µL	52.5 µL
	Red	Cas9 buffer	0.5 µL	5 µL	10 µL	15 µL
	Green	Cas9 Nuclease	0.5 µL	5 µL	10 µL	15 µL
	Purple	gRNA mix (5 µM)	2 µL	20 µL	40 µL	60 µL
		Total volume	**4.75 µL**	**47.5 µL**	**95 µL**	**142.5 µL**

3.2	Pipette-mix **RM2** up and down 10 times (do not vortex). Pipette-mix full volume.
3.3	Ensure **RM2** is fully mixed and quick-spin in a microcentrifuge to collect liquid. **Proceed to incubating the RM2 Master Mix using thermocycler program below. The full master mix volume for 8, 16, or 24 samples should be incubated in one PCR tube.**
3.4	Run the **Cas9 gRNA complex formation** thermocycler program. Set the lid temperature to 75°C.

Step	Time	Temperature
1	10 min	37°C
2	Hold	4°C

3.5	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 using Master Mix RM2 prepared in Step 3.

✔	Step	Instructions
	4.1	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 15% overage according to the table below. **RM2 is Cas9 gRNA complex from step 3 after thermocycler program.**

Reaction Mix 3 (RM3)

✔	Reagent	Component	Volume per sample	Volume per 8-plex (With 15% overage)	Volume per 16-plex (With 15% overage)	Volume per 24-plex (With 15% overage)
		NF water	12.75 µL	117.3 µL	234.6 µL	351.9 µL
	Red	Cas9 buffer	1.5 µL	13.8 µL	27.6 µL	41.4 µL
		RM2 (Cas9 gRNA complex from Step 3)	4.75 µL	43.7 µL	87.4 µL	131.1 µL
		Total volume	**19 µL**	**174.8 µL**	**349.6 µL**	**524.4 µL**

4.2	Pipette-mix **RM3** up and down 10 times (do not vortex). Pipette-mix full volume.
4.3	Quick-spin in a microcentrifuge to collect liquid.
4.4	Add **19 µL** of the RM3 to each sample from Step 2 for total reaction volume of **99 µL.**
4.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
4.6	Spin down the tube strip for 15–30 seconds in a microcentrifuge to collect liquid and remove bubbles.
4.7	Run the **Cas-9 digestion** thermocycler program. Set the lid temperature to 75°C.

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

4.8	Quick-spin in a microcentrifuge to collect liquid.
	Cleanup with 1X SMRTbell cleanup beads
4.9	Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.
4.10	Pipette-mix the beads slowly 8–10x 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.**
4.11	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 for 3–5 minutes until beads separate fully from the solution.

4.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
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 **41 µL** of **low-TE buffer** to each tube and resuspend the beads.
4.17	Quick-spin the tube strip in a microcentrifuge.
4.18	Leave at **room temperature** for **5 minutes** to elute DNA.
4.19	Place the tube strip in a magnetic separation rack for 3–5 minutes until beads separate fully from the solution.
4.20	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant 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.** **Expect recovery of 50-100% per samples relative to starting mass.**
4.22	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at – 20°C

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 in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 15% overage according to the table below.

Reaction Mix 4 (RM4)

✔	Reagent	Component	Volume per sample	Volume per 8-plex With 15% overage	Volume per 16-plex With 15% overage	Volume per 24-plex With 15% overage
		NF water	2.5 µL	23 µL	46 µL	69 µL
	Orange	dA tail buffer	5 µL	46 µL	92 µL	138 µL
	Yellow	dATP (10 mM)	1.25 µL	11.5 µL	23 µL	34.5 µL
	Light Blue	*Taq* DNA polymerase	1.25 µL	11.5 µL	23 µL	34.5 µL
		Total volume	**10 µL**	**92 µL**	**184 µL**	**276 µL**

	5.2	Pipette-mix **RM4** up and down 10 times (do not vortex). Pipette-mix full volume.
	5.3	Quick-spin **RM4** in a microcentrifuge to collect liquid.
	5.4	Add **10 µL** of the **RM4** to **40 µL** of each sample for a total reaction volume of **50 µL**.
	5.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
	5.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.7	Run the **dA-tail** thermocycler program. Set the lid temperature to 75°C.

Step	Time	Temperature
1	20 min	37°C
2	5 min	72°C
3	Hold	4°C

	5.8	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.

<table>
<tr><th>✔</th><th>Step</th><th>Instructions</th></tr>
<tr><td></td><td>6.1</td><td>To a PCR strip with 50 µL DNA from Step 5, add 4 µL SMRTbell barcoded adapter 3.0 to each sample. Tap-mix or pipette up and down 10 times (do not vortex).</td></tr>
<tr><td></td><td>6.2</td><td>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 15% overage according to the table below.

Aspirate and dispense viscous ligation mix slowly to reduce liquid sticking to the exterior and interior wall of the pipette tip.

Reaction Mix 5 (RM5)
<table><tr><th>✔</th><th>Reagent</th><th>Component</th><th>Volume per sample</th><th>Volume Per 8-plex
With 15% overage</th><th>Volume Per 16-plex
With 15% overage</th><th>Volume Per 24-plex
With 15% overage</th></tr><tr><td></td><td></td><td>NF water</td><td>2 µL</td><td>18.4 µL</td><td>36.8 µL</td><td>55.2 µL</td></tr><tr><td></td><td>Purple</td><td>Repair buffer</td><td>8 µL</td><td>73.6 µL</td><td>147.2 µL</td><td>220.8 µL*</td></tr><tr><td></td><td>Yellow</td><td>Ligation mix</td><td>30 µL</td><td>276 µL</td><td>552 µL</td><td>828 µL</td></tr><tr><td></td><td>Red</td><td>Ligation enhancer</td><td>1 µL</td><td>9.2 µL</td><td>18.4 µL</td><td>27.6 µL</td></tr><tr><td></td><td></td><td>Total volume</td><td>41 µL</td><td>377.2 µL</td><td>754.4 µL</td><td>1131.6 µL</td></tr></table>*The actual fill volume in the Repair buffer tube is >225 µL</td></tr>
<tr><td></td><td>6.3</td><td>Pipette-mix RM5 up and down 10 times (do not vortex). Pipette-mix full volume.
Ensure slow mixing and dispensing of RM5 to prevent liquid adhering to inside wall of tip and volume loss.</td></tr>
<tr><td></td><td>6.4</td><td>Quick-spin RM5 in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>6.5</td><td>Add 41 µL of RM5 to each sample from previous step for a total volume of 95 µL.</td></tr>
<tr><td></td><td>6.6</td><td>Pipette-mix each sample up and down 10 times. Pipette-mix full volume.</td></tr>
<tr><td></td><td>6.7</td><td>Quick-spin the tube strip in a microcentrifuge to collect liquid.</td></tr>
<tr><td></td><td>6.8</td><td>Run the adapter ligation thermocycler program. Set the lid temperature to 75°C.

<table><tr><th>Step</th><th>Time</th><th>Temperature</th></tr><tr><td>1</td><td>30 min</td><td>20°C</td></tr><tr><td>2</td><td>Hold</td><td>4°C</td></tr></table></td></tr>
<tr><td colspan="3">Cleanup with 1X SMRTbell cleanup beads</td></tr>
<tr><td></td><td>6.9</td><td>Add 95 µL of resuspended, room-temperature SMRTbell cleanup beads to each sample.</td></tr>
<tr><td></td><td>6.10</td><td>Pipette-mix the beads 10 times until evenly distributed.</td></tr>
</table>

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 for 3–5 minutes 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 **41 µL** of **elution buffer** to each tube and resuspend the beads.
6.19	Quick-spin the tube strip in a microcentrifuge.
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 the old tube strip with beads.
6.23	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.** **Expect recovery of 50-100% per sample relative to starting mass.**
6.23	Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at -20°C

7. Nuclease treatment

This step removes DNA fragments that have not formed SMRTbell templates. This step removes the vast majority of starting DNA fragments.

✔	Step	Instructions
	7.1	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 15% overage according to the table below.

Reaction Mix 6 (RM6)

✔	Reagent	Component	Volume per sample	Volume per 8-plex With 15% overage	Volume per 16-plex With 15% overage	Volume per 24-plex With 15% overage
	Light Purple	Nuclease buffer	5 µL	46 µL	92 µL	138 µL
	Light Green	Nuclease mix	5 µL	46 µL	92 µL	138 µL
		Total volume	**10 µL**	**92 µL**	**184 µL**	**276 µL**

	7.2	Pipette-mix **RM6** up and down 10 times (do not vortex). Pipette-mix full volume.
	7.3	Quick-spin **RM6** in a microcentrifuge to collect liquid.
	7.4	Add **10 µL** of **RM6** to **40 µL** of each sample. The total volume should equal **50 µL.**
	7.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
	7.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	7.7	Run the **nuclease treatment** thermocycler program. Set the lid temperature to 75°C.

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

	7.8	Proceed to the next step of the protocol (pooling and cleanup with 1x SMRTbell cleanup beads).

8. Sample pooling

Pool SMRTbell templates **in units of 8** samples after nuclease treatment of individual samples

✔	Step	Instructions for pooling, bead binding, washing, and sample elution
	8.1	In a 1.5 mL DNA LoBind tube combine nuclease treated libraries from step 7.7 in groups of 8 **(8 x 50 µL)** for a final sample volume of **400 µL.**
	8.2	Add **400 µL** of SMRTbell cleanup beads to each pooled nuclease treated sample.
	8.3	Pipette-mix up and down 10 times until the beads are evenly distributed. Pipette-mix full volume.
	8.4	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	8.5	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	8.6	Place the tube strip 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 1 mL of **freshly prepared 80% ethanol** into each 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 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.
	8.11	Remove the tube strip from the magnetic rack. **Immediately** add **100 µL** of **elution buffer** to each tube and resuspend the beads by pipetting up and down 10 times.
	8.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	8.14	Place the tube strip 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 **1.5 mL DNA LoBind tube**. Discard old tube with beads.
		Proceed to the next step of the protocol.

9. SMRT boost beads wash of SMRTbell templates

The SMRT boost beads wash will prepare the library for sequencing.

Wash pooled SMRTbell templates with SMRT boost beads.

Perform one SMRT boost beads wash per final pool of up to 24 samples. For >24 samples you need to perform two SMRT boost beads wash with up to 24 samples each.

Bring SMRT boost buffer and SMRT boost beads to room-temperature prior to use.

Vortex room-temperature SMRT boost buffer prior to use. Vortex room-temperature SMRT boost beads prior to use and spin down to collect.

✔	Step	Instructions
	9.1	Prepare the beads by washing in SMRT boost buffer • To a 1.5 mL DNA LoBind tube, first add 1 mL of **room-temperature SMRT boost buffer** and then 100 µL of resuspended, **room-temperature SMRT boost beads**. • Pulse-vortex 5 times to mix. • Quick-spin the tube and magnetically separate for 2 minutes until the supernatant is clear. • Remove the supernatant and wash once more by adding **1 mL of SMRT boost buffer**. • Pulse-vortex 5 times to mix. • Quick-spin the tube and magnetically separate for 2 minutes until the supernatant is clear. Remove the supernatant. • Resuspend the beads in **200 µL SMRT boost buffer** by pulse-vortexing 5 times to mix. • Quick-spin to collect the beads.
	9.2	Prepare the SMRTbell templates for SMRT boost bead washing by mixing together one or more 8-plex pools (each at 100 µL) from Step 8. Bring the total volume to **300 µL with Elution Buffer** if combining fewer than 3 pools of 8, as shown in the table below.
	9.3	Add **300 µL of SMRTbell templates** to **200 µL SMRT boost beads** in SMRT boost buffer from step 9.1. Pulse-vortex 5 times to mix. Spin down to collect.
	9.4	Gently **rotate-mix for 30 minutes at RT** using a rotator at low speed (~10 rpm).
	9.5	Spin down to collect and magnetically separate for 2 minutes until the supernatant is clear.
	9.6	Aliquot **500 µL** of the SMRTbell-containing supernatant into a fresh 1.5 mL LoBind tube.

# of Samples in Batch	8	16	24
8-plex pool 1	100 µL	100 µL	100 µL
8-plex pool 2	0 µL	100 µL	100 µL
8-plex pool 3	0 µL	0 µL	100 µL
Elution Buffer	200 µL	100 µL	0 µL
Total sample volume	300 µL	300 µL	300 µL

Cleanup with 1X SMRTbell cleanup beads

9.7	Add **500 µL** of resuspended, room-temperature SMRTbell cleanup beads to **500 µL** SMRTbell-containing supernatant from step 9.6.
9.8	Pipette-mix the beads 8–10 times until evenly distributed.
9.9	Quick–spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
9.10	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
9.11	Place the tube in a magnetic separation rack until beads separate fully from the solution.
9.12	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
9.13	Slowly dispense **1 mL** of **freshly prepared 80% ethanol** into each tube. After **30 seconds**, pipette off the 80% ethanol and discard.
9.14	Repeat the previous step.
9.15	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.
9.16	Remove the tube from the magnetic rack. **Immediately** add **100 µL** of **elution buffer** to each tube and resuspend the beads.
9.17	Leave at **room temperature** for **5 minutes** to elute DNA.
9.18	Place the tube in a magnetic separation rack until beads separate fully from the solution.
9.19	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **1.5 mL DNA LoBind tube**. Discard old tube with beads.

Proceed to the next step of the protocol.

SAFE STOPPING POINT – Store at -20°C

10. Concentrate samples with SMRTbell cleanup beads for ABC

Concentrate up to 24 SMRTbell templates into 15 µL volume for ABC.

✔	Step	Instructions
	10.1	Add **100 µL** SMRTbell cleanup beads to a 1.5mL DNA LoBind tube with **100 µL of SMRTbell templates.**
	10.2	Pipette-mix **slowly** up and down 10 times until the beads are evenly distributed.
	10.3	Quick-spin the tube in a microcentrifuge to collect all liquid from the sides of the tubes.
	10.4	Leave at **room temperature** for **10 minutes** to allow DNA to bind beads.
	10.5	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
	10.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	10.7	Slowly dispense **250 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.
	10.8	Repeat the previous step.
	10.9	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.
	10.10	Remove the tube from the magnetic rack. **Immediately** add **16 µL** of **elution buffer** to each tube and resuspend the beads.
	10.11	Leave at **room temperature** for **5 minutes** to elute DNA.
	10.12	Place the tube in a magnetic separation rack until beads separate fully from the solution.
	10.13	Slowly pipette off the cleared eluate (supernatant) without disturbing the beads. Transfer supernatant to a **1.5 mL DNA LoBind tube**. Discard old tube with beads.
	10.14	QC step: Take **1 µL** of eluted DNA for quantification and measure DNA concentration with a Qubit fluorometer using the **1X dsDNA HS kit. Do NOT use Qubit dsDNA BR assay kit as the concentration may be too low to measure. Do NOT dilute sample 1:10 as concentration may be too low to measure.** **Expect recovery of 0.5% or less (range 0.02% - 0.5%) relative to input starting mass.** For example, starting with 2 µg input per sample (or 16 µg per pool of 8 samples), the final mass recovered at this step is expected to be less than 80 ng total (range 3 ng–80 ng). Note, however, it is possible to observe recoveries outside of this range and still get good sequencing yield.
		Proceed to the next step of the protocol. **SAFE STOPPING POINT – Store at -20°C**

11a. Annealing, binding & SMRTbell cleanup (ABC) for the Revio system

Use the entire volume of 15 µL pooled SMRTbell templates per ABC preparation and loading.

Ensure each ABC preparation is per 24 samples or fewer. Thus, if processing 24 SMRTbell templates or fewer (15 µL total) go into Preparation A; If processing more than 24 SMRTbell templates, the rest of the SMRTbell templates (also pooled in 15 µL total) go into Preparation B.

✔	Step	Instructions

Note: Always use these values for each pooled preparation. Do not adjust based on measured concentration, value, or plex level.

Annealing sequencing primer

Combine the following components in a new low-binding tube and pipette to mix.

11a.1

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Sample (SMRTbell templates)	15 µL	15 µL
Annealing buffer	7.5 µL	7.5 µL
Sequencing primer	7.5 µL	7.5 µL
Total Volume	**30 µL**	**30 µL**

Incubate at room temperature for 15 minutes then proceed to the next step.

Binding sequencing polymerase

Dilute sequencing polymerase

Combine the following components in a single low-bind tube and pipette to mix. The prepared volume of diluted polymerase is sufficient to process all specified samples in a batch.

11a.2

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Polymerase stock	3.5 µL	3.5 µL
Polymerase buffer	26.5 µL	26.5 µL
Total Volume	**30 µL**	**30 µL**

Diluted polymerase must be used immediately.

Bind sequencing polymerase

Add annealed sample to diluted polymerase and finger tap or pipette to mix.

11a.3

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Annealed sample	30 µL	30 µL
Diluted Polymerase	30 µL	30 µL
Total Volume	**60 µL**	**60 µL**

Incubate at room temperature for 15 minutes. The bound complex can be stored at 4°C for 4 weeks.

Purification of polymerase bound SMRTbell complexes

1. Equilibrate the SMRTbell cleanup beads and the loading buffer to room temperature at least 30 min before use.
2. Add the following buffer volumes to each sample in each batch, as indicated:

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Binding Reaction	60 µL	60 µL
Dilution Buffer	40 µL	40 µL
Total Volume	**100 µL**	**100 µL**

3. Add the indicated volume of SMRTbell cleanup beads to each sample in each preparation and gently pipette-mix. Incubate on the benchtop for 10 minutes.

11a.4

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Binding Reaction	100 µL	100 µL
SMRTbell cleanup Beads	120 µL	120 µL
Total Volume	**220 µL**	**220 µL**

4. Place each tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. Discard the supernatant. **DO NOT** wash the collected bead pellet with ethanol.
5. Immediately resuspend the beads in the indicated volumes of room temperature Loading buffer and pipette-mix:

	Preparation A	Preparation B
# of samples in preparation	8 or 16 or 24	8 or 16 or 24
Loading Buffer	49 µL	49 µL

6. To elute the polymerase-bound complexes, incubate the samples on the benchtop for at least 10 minutes at room temperature.
7. Place each tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.
8. Transfer eluates to new low-binding tube. Place on ice **and protect from light**.

Internal control dilution

Prepare only one control dilution, regardless of number of samples (up to 48 samples).

11a.5

1st Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

Reagent	Internal control
Dilution buffer	19 µL
Sequencing control	1.0 µL
Total volume	20 µL

2nd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

11a.6

Reagent	Internal control
Dilution buffer	19 µL
Sequencing control (dilution 1)	1.0 µL
Total volume	20 µL

3rd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

11a.7

Reagent	Internal control
Dilution buffer	19 µL
Sequencing control (dilution 2)	1.0 µL
Total Volume	20 µL

Final loading dilution

Combine the following and **protect from light**:

If loading ≤24 samples

11a.8

# of samples in preparation	≤24-plex
Prepared sample (preparation A)	48.5 µL
Loading buffer	48.5 µL
Diluted internal control (dilution 3)	3 µL
Total volume	100 µL

Load 100 µL of sample per well and/or store at 4C for up to 24 hours before use.

If loading >24 samples

# of samples in preparation	>24-plex
Prepared sample (preparation A)	48.5 µL
Prepared sample (preparation B)	48.5 µL
Loading buffer	0 µL
Diluted internal control (dilution 3)	3 µL
Total volume	100 µL

Load 100 µL of sample per well and/or store at 4°C for up to 24 hours before use.

11b. Annealing, binding, & SMRTbell cleanup (ABC) for Sequel II systems

Use entire volume of 15 µL pooled SMRTbell templates per ABC batch and loading.

✔	Step	Instructions

Note: Always use these values for each pooled batch. Do not adjust based on measured concentration, value, or plex level of batch.

Annealing sequencing primer

Combine the following components in a new low-binding tube and pipette to mix.

11b.1

# of samples in batch	8 or 16 or 24
Sample (SMRTbell templates)	15 µL
Annealing Buffer	7.5 µL
Sequel II Primer 3.2	7.5 µL
Total volume	**30 µL**

Incubate at room temperature for 15 minutes then proceed to the next step.

Binding sequencing polymerase

Dilute sequencing polymerase

Combine the following components in a single low-bind tube and pipette to mix. The prepared volume of diluted polymerase is sufficient to process all specified samples in a batch.

11b.2

# of samples in batch	8 or 16 or 24
Polymerase Stock	3.5 µL
Sequel II polymerase dilution buffer	26.5 µL
Total Volume	**30 µL**

Diluted polymerase must be used immediately.

Bind sequencing polymerase

Add annealed sample to diluted polymerase and finger tap or pipette to mix.

11b.3

# of samples in Batch	8 or 16 or 24
Annealed sample	30 µL
Diluted polymerase	30 µL
Total volume	**60 µL**

Incubate at room temperature for 15 minutes. Bound complex can be stored at 4°C for 4 weeks.

Purification of polymerase bound SMRTbell complexes

1. Equilibrate the cleanup beads and Sequel II loading buffer 3.2 to room temperature at least 30 min before use.
2. Add the following buffer volumes to each sample in each batch, as indicated:

# of samples in batch	8 or 16 or 24
Binding reaction	60 µL
ABC buffer	40 µL
Total volume	**100 µL**

3. Add the indicated volume of cleanup beads to each sample in each batch and gently pipette-mix. Incubate on the benchtop for 10 minutes.

# of samples in batch	8 or 16 or 24
Binding reaction	100 µL
Cleanup beads	120 µL
Total volume	**220 µL**

11b.4

4. Place each tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. Discard the supernatant. **DO NOT** wash the collected bead pellet with ethanol.
5. Immediately resuspend the beads in the indicated volumes of room temperature Sequel II loading buffer 3.2 and pipette-mix:

# of samples in batch	8 or 16 or 24
Sequel II loading buffer 3.2	50 µL

6. To elute the polymerase-bound complexes, incubate the samples on the benchtop for at least 10 minutes at room temperature.
7. Place each tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear.
8. Transfer eluates to new low-binding tube. Place on ice **and protect from light**.

Internal control dilution

1st Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

11b.5

Reagent	Internal control
ABC buffer	19 µL
Sequel II DNA internal control complex 3.2	1.0 µL
Total volume	**20 µL**

11b.6 2nd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

Reagent	Internal control
ABC buffer	19 µL
Sequel II DNA internal control complex 3.2 (dilution 1)	1.0 µL
Total volume	20 µL

11b.7

3rd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

Reagent	Internal control
ABC buffer	19 µL
Sequel II DNA internal control complex 3.2 (dilution 2)	1.0 µL
Total volume	20 µL

Final loading dilution

11b.8

Combine the following and **protect from light**:

# of samples in batch	Sample 1 (24-plex or less)
Prepared sample	50 µL
Sequel II loading buffer 3.2	67 µL
Diluted internal control (dilution 3)	3 µL
Total volume	120 µL

Load 115 µL of sample per well and/or store at 4°C for up to 24 hours before use.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	March 2024
Minor updates for clarity	02	April 2024

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