Ampiifying genomic DNA for SMRTbell library preparation and HiFi sequencing

Procedure & checklist

PacBio

Overview

This procedure describes the workflow for constructing SMRTbell® libraries with PCR amplification from as little as 1 ng of starting genomic DNA using the Ampli-Fi protocol. Sequencing libraries prepared with this procedure are compatible with PacBio® Revio® and Vega™ systems.

Protocol overview			
Application	Genome sequencing	This protocol is intended for samples where there is insufficient DNA for PCR-free library prep, where difficult-to-remove sequencing inhibitors may be present, or where there is extensive DNA damage present	
Genomic DNA Input	1–50 ng per sample	Recommended starting double-stranded DNA input amount for protocol	
Target DNA fragment size	7–11 kb	Average fragment size of sheared DNA to balance read length with amplification efficiency	
PCR polymerase	KOD Xtreme Hot Start DNA polymerase	Minimizes GC coverage bias with efficient amplification	
Amplification adapter	Twist Universal Adapter System	Common adapter for NGS that enables unique dual-indexed (UDI) barcoding prior to SMRTbell library prep	
SMRTbell library prep	SMRTbell prep kit 3.0	Supports the ligation of amplification adapters and the SMRTbell library preparation of amplified DNA for up to 24 samples	
		Revio (SPRQ™ chemistry)	**Revio (non-SPRQ chemistry_/Vega**
Required mass of amplified DNA into library prep per SMRT® Cell		150 ng	600 ng
		Time	
Protocol time from gDNA shearing to SMRTbell library		≤7 hr	
Protocol time for ABC workflow (polymerase binding)		1 hr	

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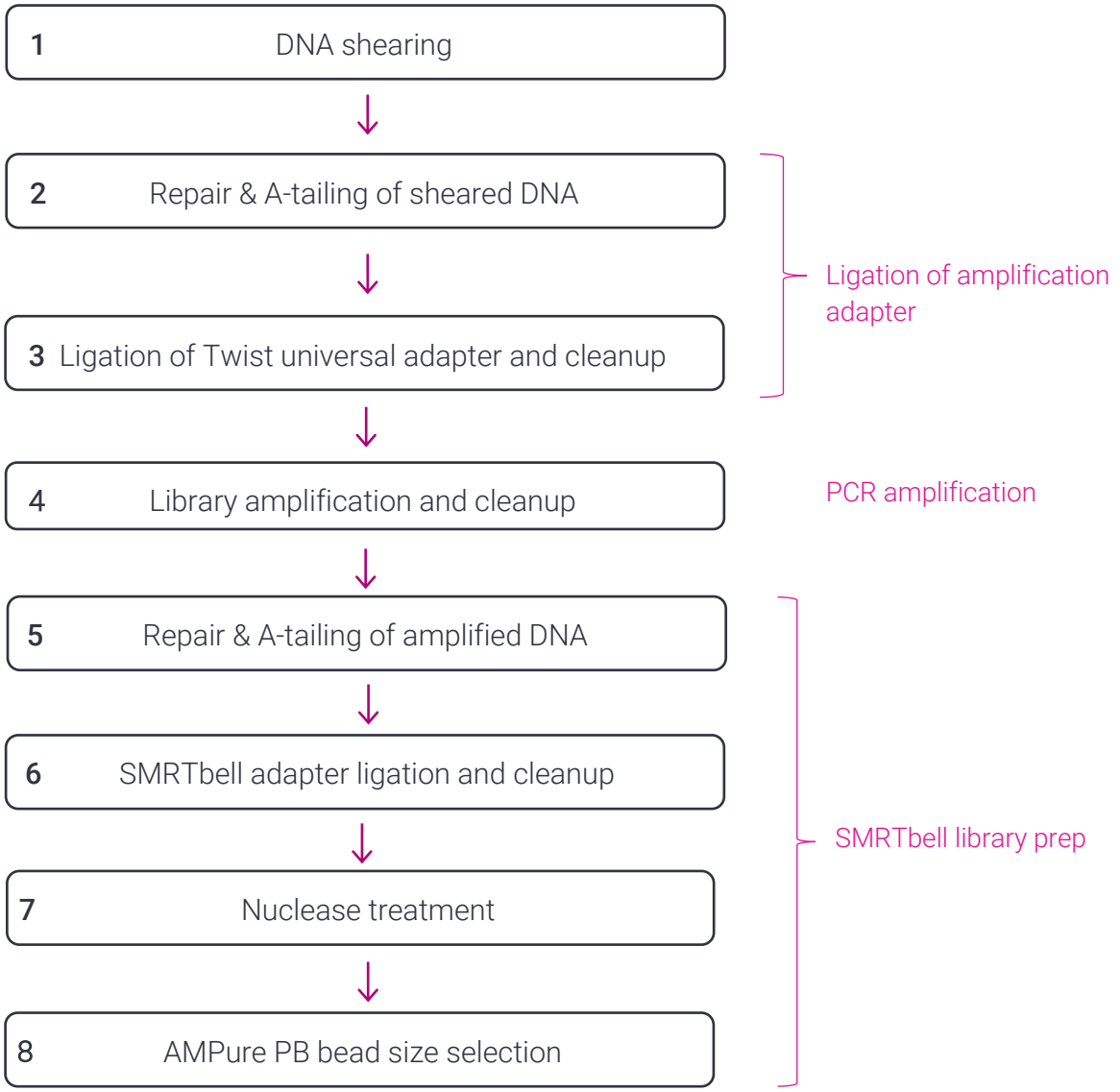
Workflow overview



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Required materials and equipment

DNA quantification	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
DNA shearing	
Megaruptor 3	Diagenode B06010003
Megaruptor 3 shearing kit	Diagenode E07010003
or	
g-TUBE	Covaris 520104
PCR amplification and SMRTbell® library preparation	
KOD Xtreme™ Hot Start DNA polymerase (200U) (outside of Japan) KOD FX (200U) (Japan)	Millipore Sigma 71975-3* TOYOBO KFX-101
Twist Universal Adapter System	Twist 101307-101311
SMRTbell® prep kit 3.0, includes: • SMRTbell® prep kit 3.0 • SMRTbell® cleanup beads • Low TE buffer	PacBio® 102-182-700
AMPure® PB bead size selection kit	PacBio® 102-182-500
Revio® SPRQ™ polymerase kit ***or*** Vega™ polymerase kit ***or*** Revio® polymerase kit (non-SPRQ™)**	PacBio® 103-496-900 PacBio® 103-426-500 PacBio® 102-739-100
*Manufactured by Toyobo, Japan and distributed by Merck KGaA globally. **Procedure for Revio polymerase kit (non-SPRQ) can be found in SMRT® Link Sample Setup	
Other common lab supplies	
100% ethanol, molecular biology	Any major lab supplier (MLS)
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Magnetic separation rack compatible with 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
Thermocycler compatible with 100 µL and 8-tube strips	Any MLS
Microcentrifuge	Any MLS

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Before you begin

The following are best practices for preparing SMRTbell libraries using PCR for HiFi sequencing on Revio or Vega systems. Please read carefully prior to beginning the procedure.

Application

This procedure is intended for, but not limited to, the following sample types:

- Those that do not have sufficient DNA mass for PCR-free sequencing (e.g. single, small insect)
- Samples contaminated with sequencing inhibitors that are challenging to remove (e.g., snails or marine organisms)
- Preserved specimens collected in the field (e.g., ethanol preserved tissue)
- Chromosomal conformation capture (3C) samples (e.g., CiFi application)
- DNA from FFPE tissue (please note that DNA quantity and quality is highly variable from FFPE)

The mean library size is expected to be less than 10 kb due to the limitations of PCR. Please note that methylation signals will not be retained with this application.

Genomic DNA (gDNA) QC and input amount recommendations

Sequencing yield expectations should be set in accordance with the genomic DNA quality. Samples with higher amounts of degraded or fragmented DNA will have lower average inserts sizes due to preferential amplification of the shorter fragments. To maximize HiFi base yield and average read length, it is recommended to start with HMW DNA. If longer HiFi read lengths are required for more degraded samples, pre- or post-library size selection methods may be used.

gDNA quality QC

The Agilent Femto Pulse system is highly recommended for accurately measuring the distribution gDNA mass. Please see the PacBio Technical note for more details.

Recommended guidelines for evaluating gDNA quality for this protocol:

- Use the Femto Pulse gDNA 165 kb analysis kit (Agilent FP-1002-0275)
- Dilute aliquot of the sample to 250 pg/µL

gDNA input mass

The supported genomic DNA input mass for this protocol ranges from 1 to 50 ng. The number of PCR cycles required will depend on the input mass.

Size selection of SMRTbell library

This protocol uses the diluted 3.1X (35% v/v) AMPure PB size selection and cleanup method to progressively deplete DNA below 5 kb. For libraries with an average size less than 5 kb, a standard 1X SMRTbell bead cleanup is recommended (e.g., FFPE samples)

Gel size selection approaches can be used to enrich for larger insert sizes when there is sufficient library available. Please see this Technical Note on gel cassette size selection.

Multiplexing

Please visit our multiplexing webpage for the demultiplexing fasta file to import into SMRT Link versions 25.1 and above. We recommend using this file for demultiplexing and adapter trimming.

Reagent and sample handling

Room temperature is defined as any temperature in the range of **18–25°C** for this protocol.

SMRTbell cleanup beads and AMPure PB beads

Bring SMRTbell cleanup beads and AMPure PB beads to room temperature prior to use.

Vortex *immediately* before any addition to sample. Failure to do this will result in low recovery.

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

SMRTbell prep kit 3.0

Thaw the Repair buffer, Nuclease buffer, and SMRTbell adapter at room temperature. Mix reagent buffers with a brief vortex prior to use.

Thaw Elution buffer at room temperature. Elution buffer can be left at room temperature long term after thawing.

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

Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents	
Reagent	**Tube color**
End repair mix	Blue
DNA repair mix	Green
SMRTbell adapter	Orange
Ligation mix	Yellow
Ligation enhancer	Red
Nuclease mix	Light green

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.

Keep the following reagents on a cold block or ice:

- Sequencing polymerase
- Sequencing control

Bring the following reagents to room temperature 30 minutes prior to use:

- Loading buffer

Third party reagents

Thaw 2x Xtreme buffer, dNTP, Twist universal adapter and UDI primers at room temperature.

Safety precautions

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

Workflow steps

1. DNA shearing

We recommend shearing to a size range of 7–11 kb using either Diagenode Megaruptor 3 system (option 1) or Covaris g-TUBE (option 2). An example of the recommended shearing size distribution is shown in Figure 1.

Option 1: DNA shearing with the Megaruptor 3 system

✔	Step	Instructions
	1.1	Bring 1–50 ng DNA up to a final volume of **65 µL** with low TE buffer.
	1.2	Shear DNA on the Megaruptor 3 system with the following parameters: Shear speed: 59 — Target insert length: 10 kb **Note:** It is recommended to confirm gDNA is sheared to the appropriate size range (<11 kb) prior to proceeding. If the DNA is under-sheared, a second shear with the same parameters can be repeated. The same Megaruptor consumables can be used if a secondary shear is required.
	1.3	Transfer sheared DNA into a tube strip or other appropriate tube for the Repair and A tail step. Typical volume loss during shearing is between 5–10 µL.
	1.4	Recommended: evaluate sample quality (concentration and size distribution). • Take a 1–2 µL aliquot and measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Measure DNA size distribution with a Femto Pulse system. If the DNA size is >11 kb, repeat step 1.2 (see note).

Option 2: DNA shearing with a Covaris g-TUBE

✔	Step	Instructions
	1.1	Dilute 1–50 ng of gDNA in a final volume of **65 µL** with low TE buffer.
	1.2	Transfer gDNA to the g-TUBE and centrifuge at **2348 x *g* for 5 minutes** to achieve a target mode of 10 kb.
	1.3	Check for any residual sample remaining in the upper chamber of the g-TUBE. If present, re-spin for 1 minute. Repeat spin until the entire gDNA sample has passed through the orifice.
	1.4	Invert and spin the g-TUBE at the same speed selected in step 1.2 until the entire gDNA sample has passed through the g-TUBE orifice.
	1.5	Transfer the recovered sheared DNA to a new 0.2 mL 8-tube strip. Up to 10% volume loss is typical. **Note:** It is acceptable to proceed with up to 60 µL of sample into the Repair and A-tailing step.

SAFE STOPPING POINT - Store at 4°C

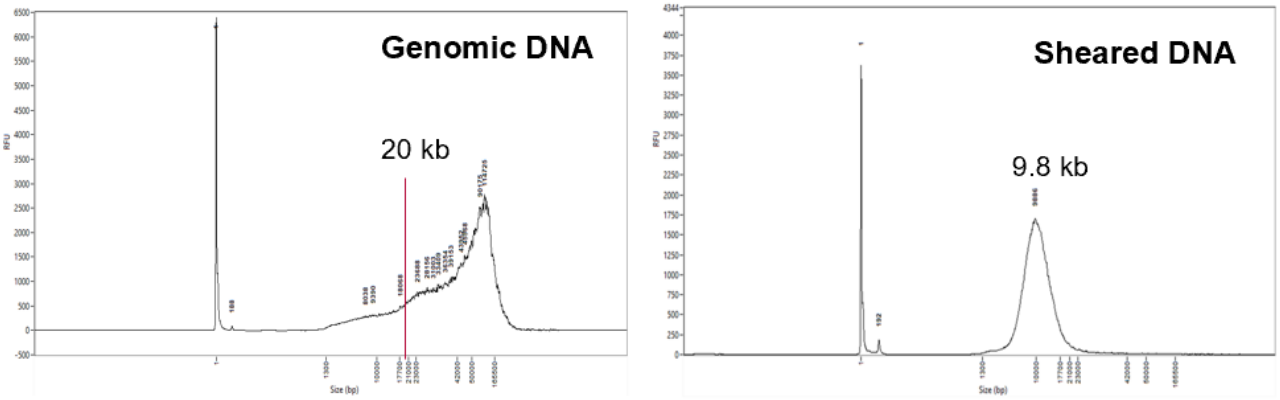

Figure 1. Example of 5 ng of human genomic DNA sheared to approximately 10 kb using the Covaris g-TUBE. Distribution of starting genomic DNA is >20 kb.

2. Repair and A-tailing of sheared DNA

This step repairs and prepares the DNA for A-T ligation.

✔	Step	Instructions

2.1 Add the following components from the SMRTbell prep kit 3.0 to a new microcentrifuge tube. Adjust component volumes for the number of samples being amplified, plus 15% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps 2.2 to 2.4.

Repair master mix

✔	Tube	Component	Volume		
			Per library	4 libraries*	8 libraries*
	Purple	Repair buffer	8 µL	36.8 µL	73.6 µL
	Blue	End repair mix	2 µL	9.2 µL	18.4 µL
	Green	DNA repair mix	1 µL	4.6 µL	9.2 µL
		Total volume	**11 µL**	**50.6 µL**	**101.2 µL**

*15% overage included in master mix calculations

2.2 Pipette-mix the Repair master mix.

2.3 Quick-spin the Repair master mix in a microcentrifuge to collect liquid.

2.4 Add 11 µL of the Repair master mix to each sample. Total reaction volume should be 60 µL.

Note: It is acceptable to use up to 60 µL of sample (post-shearing) without negatively impacting enzymatic or cleanup reactions. Recovery sample volume from Megaruptor is typically 50–60 µL.

2.5 Pipette-mix each sample.

2.6 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

2.7 Run the Repair and 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

2.8 Proceed to the next step of the protocol.

3. Ligation of amplification adapter and cleanup

This step ligates the amplification adapter required for the PCR step.

✔	Step	Instructions

3.1 Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of libraries 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 steps 3.2 to 3.4.

Ligation master mix					
✔	Tube	Component	Volume		
			Per library	4 libraries*	8 libraries*
		Twist Universal Adapter	2 µL	8.8 µL	17.6 µL
	Yellow	Ligation mix	15 µL	66 µL	132 µL
	Red	Ligation enhancer	1 µL	4.4 µL	8.8 µL
		Total volume	**18 µL**	**79.2 µL**	**158.4 µL**

*10% overage included in master mix calculation

3.2 Pipette-mix the Ligation master mix.

3.3 Quick-spin the Ligation master mix in a microcentrifuge to collect liquid.

3.4 Add **18 µL** of the Ligation master mix containing the Twist universal adapter to each sample from the previous step.

The total volume per sample should be **78 µL**.

3.5 Pipette-mix each sample.

3.6 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

3.7 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 1X SMRTbell cleanup beads

3.8 Add **78 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.

3.9 Pipette-mix the beads until evenly distributed.

3.10 Quick-spin the sample(s) in a microcentrifuge to collect all liquid from the sides of the tubes.

3.11 Incubate at room temperature for 10 minutes to allow DNA to bind beads.

3.12 Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.

3.13 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

3.14 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.

3.15 Repeat the previous step.

3.16 Remove residual 80% ethanol:

- Remove the sample(s) from the magnetic separation rack.
- Quick-spin the sample(s) in a microcentrifuge.
- Place the sample(s) back in a magnetic separation rack until beads separate fully from the solution.
- Remove residual 80% ethanol and discard.

3.17 Remove the sample(s) from the magnetic rack. Immediately add **24 µL of Elution buffer** to each tube and resuspend the beads.

3.18 Quick-spin the sample(s) in a microcentrifuge.

3.19 Incubate at room temperature for 5 minutes to elute DNA.

3.20 Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.

3.21 Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube. Discard old tube(s) with beads.

3.22 Proceed to the next step of the protocol.

4. Amplification and cleanup

This step amplifies DNA ligated with the amplification adapter from the previous step.

✔	Step	Instructions
	4.1	Add the following components to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. **Add 4 µL of Twist UDI primer to each sample.** For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps 4.2 to 4.4.
	4.2	Pipette-mix the Amplification master mix.
	4.3	Quick-spin the Amplification master mix in a microcentrifuge to collect liquid.
	4.4	On ice, add **72 µL** of the Amplification master mix to **28 µL of sample** and primer for a total volume of 100 µL**.**
	4.5	Pipette-mix.
	4.6	Quick-spin the sample(s) in a microcentrifuge to collect liquid.

Amplification master mix

✔	Component	Volume		
		Per library	4 libraries*	8 libraries*
	2x Xtreme buffer	50 µL	220 µL	440 µL
	dNTP (2 mM each)	20 µL	88 µL	176 µL
	KOD Xtreme Hot Start DNA polymerase	2 µL	8.8 µL	17.6 µL
	Twist UDI Primers (plate)	4 µL		
	Total volume	**76 µL**	**316.8 µL**	**633.6 µL**

*10% overage included in master mix calculation

Run the PCR thermocycler program with the lid temperature set to 105°C. Do not add samples to thermal cycler until lid has pre-heated.

Step	Time	Temperature	Cycles
1	2 min	94°C	1 cycle
2	10 sec	98°C	8–14 cycles
3	30 sec	58.8°C	
4	10 min	68°C	
5	7 min	68°C	1 cycle
6	Hold	4°C	

4.7

gDNA input	PCR Cycles*
1 ng	14 cycles
5 ng	12 cycles
10 ng	11 cycles
20 ng	10 cycles
50 ng	8 cycles

*PCR cycles may require optimization

Cleanup with 1X SMRTbell cleanup beads

4.8 Add 100 µL of resuspended, room-temperature SMRTbell cleanup beads to each sample.

4.9 Pipette-mix the beads until evenly distributed.

4.10 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

4.11 Incubate at room temperature for 10 minutes to allow DNA to bind beads.

4.12 Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.

4.13 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

4.14 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.

4.15 Repeat the previous step.

4.16 Remove residual 80% ethanol:

- Remove the sample(s) from the magnetic separation rack.

- Quick-spin the sample(s) in a microcentrifuge.
- Place the sample(s) back in a magnetic separation rack until beads separate fully from the solution.
- Remove residual 80% ethanol and discard.

4.17 Remove the sample(s) from the magnetic rack. Immediately add **50 µL of Elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.

4.18 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

4.19 Incubate at room temperature for 5 minutes to elute DNA.

4.20 Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.

4.21 Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube. Discard old tube(s) with beads.

4.22 Recommended: Evaluate sample quality (concentration and size distribution).

- Take a 1 µL aliquot from each tube and dilute with 9 µL of Elution buffer or water.
- Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
- Dilute each aliquot to 250 pg/µL in Femto Pulse dilution buffer.
- Measure DNA size distribution with a Femto Pulse system.

IMPORTANT: The SMRTbell library preparation requires at least 150 ng of amplified DNA for sequencing on a Revio SMRT Cell using SPRQ chemistry. Sequencing on a Vega or Revio (non-SPRQ) SMRT Cell requires at least 600 ng of amplified DNA. Please see the Appendix for guidance of additional PCR cycles.

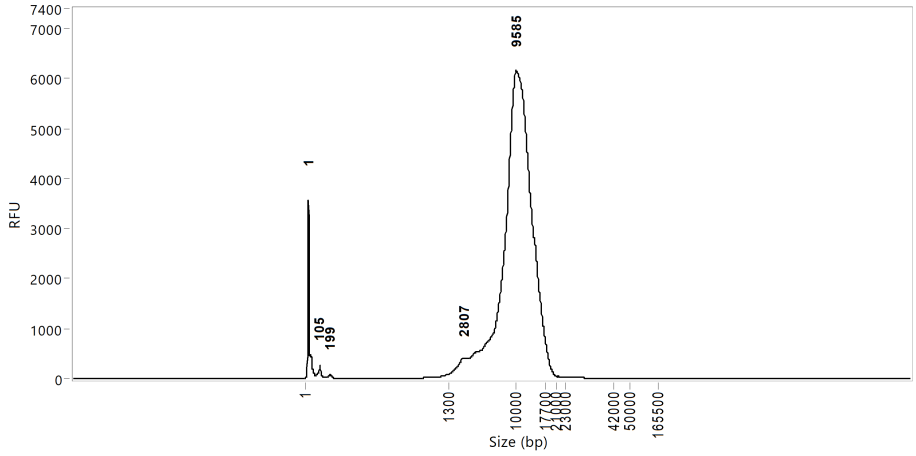

Figure 2. Example of human sample amplified by PCR. Size distribution of amplified products is approximately 10 kb and appropriate to proceed to SMRTbell library construction

4.23 Proceed to the next step of the protocol if sample quality and mass is acceptable.

SAFE STOPPING POINT - Store at 4°C

5. Repair and A-tailing of amplified DNA

If multiplexing, indexed amplified samples can be pooled at this stage prior to SMRTbell library preparation with SMRTbell prep kit 3.0 to save on costs and time. Alternatively, samples can be pooled at the end of the protocol prior to sequencing.

This step will prepare the amplified DNA from the previous step for SMRTbell adapter ligation.

✔	Step	Instructions

5.1 Add the following components from the SMRTbell prep kit 3.0 to a microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 15% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps 5.2 to 5.4.

Repair master mix

✔	Tube	Component	Volume		
			Per library	4 libraries*	8 libraries*
	Purple	Repair buffer	8 µL	36.8 µL	73.6 µL
	Blue	End repair mix	2 µL	9.2 µL	18.4 µL
	Green	DNA repair mix	1 µL	4.6 µL	9.2 µL
		Total volume	**11 µL**	**50.6 µL**	**101.2 µL**

*15% overage included in master mix calculations

5.2 Pipette-mix the Repair master mix.

5.3 Quick-spin the Repair master mix in a microcentrifuge to collect liquid.

5.4 Add **11 µL** of the Repair master mix to **49 µL of sample** for a total volume of 60 µL.

5.5 Pipette-mix each sample.

5.6 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

5.7 Run the Repair and 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.

6. SMRTbell adapter ligation and cleanup

This step ligates the SMRTbell adapter to the ends of each DNA fragment. Samples have already been indexed with the Twist UDIs during the amplification step and therefore do not require the use of a SMRTbell adapter index for demultiplexing. However, if desired, SMRTbell adapter indexes can be used from one of the four SMRTbell adapter index plates 96(A, B, C or D). The latter will require two separate demultiplexing steps when processing the sequencing data.

✔	Step	Instructions
		Proceed to step 6.2 if not using a SMRTbell adapter index from plates 96(A, B, C, or D).
	6.1	**Optional dual indexing:** Add 4 µL of the indexed adapter from the SMRTbell adapter index plate 96(A, B, C, or D) to each respective sample from the previous step and exclude the SMRTbell adapter from the Ligation master mix (next step). One index per SMRTbell adapter index plate well per sample.
	6.2	To make the Ligation master mix, add the following components from SMRTbell prep kit 3.0 to a microcentrifuge tube. (Exclude the SMRTbell adapter if using an indexed adapter from the previous step.) Adjust component volumes for the number of libraries 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 steps 6.3 to 6.5.

Ligation master mix

✔	Tube	Component	Volume		
			Per library	4 libraries**	8 libraries**
		SMRTbell adapter*	4 µL	17.6 µL	35.2 µL
	Yellow	Ligation mix	15 µL	66 µL	132 µL
	Red	Ligation enhancer	1 µL	4.4 µL	8.8 µL
		Total volume	**20 µL**	**88 µL**	**176 µL**

* Exclude the SMRTbell adapter if using the SMRTbell adapter index plate 96 (A, B, C, or D)
** 10% overage included in mastemix calculation.

	6.3	Pipette-mix the Ligation master mix.
	6.4	Quick-spin the Ligation master mix in a microcentrifuge to collect liquid.
	6.5	• **Standard, non-indexed SMRTbell adapter**: add **20 µL** of the Ligation master mix containing the SMRTbell adapter to each sample. • **SMRTbell adapter index**: add **16 µL** of the Ligation master mix containing to each sample. The total volume per sample should be **80 µL**.
	6.6	Pipette-mix each sample.
	6.7	Quick-spin the sample(s) in a microcentrifuge to collect liquid.

6.8 Run the Adapter ligation thermocycler program.

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

Cleanup with 1X SMRTbell cleanup beads

6.9 Add 80 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 sample(s) 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 sample(s) 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.

6.17 Remove residual 80% ethanol:

- Remove the sample(s) from the magnetic separation rack.
- Quick-spin the sample(s) in a microcentrifuge.
- Place the sample(s) back in a magnetic separation rack until beads separate fully from the solution.
- Remove residual 80% ethanol and discard.

6.18 Remove the sample(s) from the magnetic rack. Immediately add **40 µL of Elution buffer** to each tube and resuspend the beads.

6.19 Quick-spin the sample(s) in a microcentrifuge.

6.20 Incubate at room temperature for 5 minutes to elute DNA.

6.21 Place the sample(s) 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. Discard old tube(s) with beads.

6.23 Proceed to the next step of the protocol.

SAFE STOPPING POINT - Store at 4°C

7. Nuclease treatment of SMRTbell library

This step removes un-ligated DNA fragments and leftover adapter from the library.

✔	Step	Instructions
	7.1	Add the following components from the SMRTbell prep kit 3.0 to a new microcentrifuge tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step and skip steps 7.2 to 7.4.

Nuclease master mix

✔	Tube	Component	Volume		
			Per library	4 libraries*	8 libraries*
	Light purple	Nuclease buffer	5 µL	22 µL	44 µL
	Light green	Nuclease mix	5 µL	22 µL	44 µL
		Total volume	**10 µL**	**44 µL**	**88 µL**

7.2	Pipette-mix Nuclease master mix.
7.3	Quick-spin the 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 sample(s) in a microcentrifuge to collect liquid.
7.7	Run the Nuclease treatment thermocycler program with the lid temperature set to >47°C.

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

7.8	Proceed to the next step of the protocol. It is necessary to perform a bead cleanup on the nuclease reaction prior to safely storing the library or libraries.

8. Diluted AMPure PB bead cleanup and size selection

The AMPure PB bead size selection step progressively depletes DNA fragments less than 5 kb. Ensure accurate ratios are maintained when diluting AMPure PB and when adding the dilution to the library. Failure to do this will result in a loss of sample, or ineffective size-selection.

Important: If DNA size range is <5 kb, proceed with a 1x SMRTbell bead cleanup.

✔	Step	Instructions
	8.1	Make a 35% v/v dilution of AMPure PB beads by adding 437.5 µL of resuspended AMPure PB beads to 812.5 µL of Elution buffer. The 35% dilution can be stored at 4°C for 30 days. **Note:** The AMPure PB dilution may be scaled as appropriate for smaller/larger scale projects.
	8.2	Add 3.1X v/v (155 µL) of resuspended, room-temperature 35% AMPure PB beads to each sample from the previous step.
	8.3	Pipette-mix the beads until evenly distributed.
	8.4	Incubate at room temperature for 20 minutes to allow DNA to bind beads.
	8.5	Place sample on an appropriate magnet and allow beads separate fully from the solution.
	8.6	Slowly remove the cleared supernatant without disturbing the beads.
	8.7	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each sample. After 30 seconds, remove the 80% ethanol and discard.
	8.8	Repeat the previous step.
	8.9	Remove residual 80% ethanol: • Remove the sample from the magnet. • Quick spin to collect liquid at the bottom. • Place sample back on the magnet and allow beads separate fully from the solution. • Remove residual 80% ethanol and discard.
	8.10	Remove samples from the magnet and immediately add **25 µL of Elution buffer** to each sample.
	8.11	Pipette-mix the beads until evenly distributed.
	8.12	Incubate at room temperature for 5 minutes to elute DNA of the beads.
	8.13	Place samples on the magnet and allow the beads to separate fully from the solution.
	8.14	Slowly remove the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube.

8.15 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 final overall recovery should be 20-40% as measured from post PCR cleanup to completed SMRTbell library. **DNA concentration must be less than 20 ng/µL to proceed to ABC.**

Recommended: Further dilute each aliquot to 250 pg/µL with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.

8.16 **If required, dilute 25 µL of library to less than 20 ng/µL if in the 3–10kb size range. If DNA size is less than 3 kb, dilute to less than 10 ng/µL.** If library concentration is higher than recommended for ABC, sequencing performance will be compromised.

8.17 Proceed to Section 9 to prepare library for sequencing with Revio +SPRQ or Vega

Or

Proceed to SMRT Link Sample Setup for preparing samples for Revio non-SPRQ chemistry or Sequel II/e.

SAFE STOPPING POINT - Store at 4°C for up to 2 weeks or at -20°C long term

9. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 µL) for sequencing on Revio with SPRQ or Vega. If samples are pooled prior to ABC or a custom volume is required, see Appendix A2. **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	Ensure samples are in the correct concentration range (see 8.16). Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below. For individual preps, add components directly to each sample from the previous step (8.16) and skip steps 9.2 to 9.4.

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.
	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 color	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 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.

Post-binding cleanup with 1X SMRTbell cleanup beads

9.13 Add **100 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	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 **15 minutes** to elute DNA

9.21 Place sample on magnet and allow beads to separate fully from the solution.

9.22 Slowly aspirate 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:

- Revio is 120–160 pM.
- Vega is 100–140 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.

A1. Appendix –additional amplification for samples with low PCR yield

If there is not enough DNA to proceed with library construction, this section describes a workflow for additional DNA amplification by PCR.

✔	Step	Instructions

A1.1 Add the following components from the KOD Xtreme Hot Start DNA polymerase, plus the Twist UDI primers in the order and volume listed below to a new PCR tube. Adjust component volumes for the number of libraries being prepared, plus 10% overage. For individual preps, add components directly to the sample from the previous step at the specified volumes and skip steps (A1.2 to A1.4).

PCR mix	
Component	Per sample vol.
2x Xtreme buffer	50 µL
dNTP (2mM ea.)	20 µL
KOD Xtreme Hot Start DNA polymerase	2 µL
Twist UDI Primers	4 µL
Purified, amplified DNA (step 5.22)	24 µL
Total volume	100 µL

A1.2 Pipette-mix the PCR mix. Quick-spin the PCR mix in a microcentrifuge to collect liquid.

A1.3 Mix 24 uL purified, amplified DNA with 76 µL of the PCR mix. Total reaction volume should be 100 µL.

A1.4 Pipette-mix each sample.

A1.5 Quick-spin the sample(s) in a microcentrifuge to collect liquid.

A1.6 Run the PCR thermocycler program. (Lid: 105°C).

Step	Time	Temperature	Cycles
1	2 min	94°C	1 cycle
2	10 sec	98°C	N* cycles (See below)
3	10 min	68°C	
4	7 min	68°C	1 cycle
5	Hold	4°C	

Additional # of cycles	Condition
1	If the total mass <250 ng (<5 ng/uL)
2	If the total mass <140 ng (<2.8 ng/uL)
3	If the total mass <80 ng (<1.6 ng/µL)

Cleanup with 1X SMRTbell cleanup beads

A1.7 Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample.

A1.8	Pipette-mix the beads until evenly distributed.
A1.9	Quick-spin the sample(s) in a microcentrifuge to collect all liquid from the sides of the tubes.
A1.10	Incubate at room temperature for **10 minutes** to allow DNA to bind beads.
A1.11	Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.
A1.12	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
A1.13	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.
A1.14	Repeat the previous step.
A1.15	Remove residual 80% ethanol: • Remove the sample(s) from the magnetic separation rack. • Quick-spin the sample(s) in a microcentrifuge. • Place the sample(s) back in a magnetic separation rack until beads separate fully from the solution. • Remove residual 80% ethanol and discard.
A1.16	Remove the sample(s) from the magnetic rack. Immediately add **50 µL** of Elution buffer to each tube and resuspend the beads.
A1.17	Quick-spin the sample(s) in a microcentrifuge.
A1.18	Incubate at room temperature for **5 minutes** to elute DNA.
A1.19	Place the sample(s) in a magnetic separation rack until beads separate fully from the solution.
A1.20	Slowly remove the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube. Discard old tube(s) with beads.
A1.21	Use 1 µL of reamplified samples to quantify with Qubit dsDNA HS kit.

The re-amplified DNA can be stored at 4°C or at -20°C for future use.

A2. 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. For libraries < 3kb in size, dilute to <10 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.

FAQs

1. **Does this protocol work with FFPE samples?**
    - Ampli-Fi has been successfully executed with FFPE tissue and cell samples and achieves a significantly higher HiFi yield than PCR-free sequencing. A few points to consider are:
        - If not already extracted, we recommend using the QIAamp DNA FFPE Tissue Kit for DNA Extraction and following the Qiagen Supplementary Protocol, which uses Deparaffinization Solution (*cat. No. 19093*).
        - FFPE tissue samples generally do not require shearing. We recommend sizing with Agilent Femto Pulse or TapeStation Genomic DNA ScreenTape. If shearing is required, follow the standard shearing guidelines described in the protocol.
        - For FFPE tissue, the HiFi read length ranges between 800–2 kb in size.
        - For DNA larger than 5 kb, follow the protocol for diluted AMPure size selection (Section 8) for the final SMRTbell cleanup.
        - For DNA between 3–5 kb, perform a 1x bead cleanup for the final SMRTbell cleanup. Diluted AMPure size selection is not recommended and will result in sample loss.
        - For DNA smaller than 3 kb, replace all 1x bead cleanups with 1.3x bead cleanups.
        - To increase the size of fragmented FFPE samples (<3 kb), the post-PCR SMRTbell bead cleanup can be adjusted to be more stringent (1x); however, this will be sample–dependent. If attempting a stringent size-selection, we recommend increasing the number of PCR cycles to account for loss.

2. **What should I do if my DNA is under-sheared on the Megaruptor?**
    - If the Femto Pulse shows a shear mode >11 kb, we recommend performing an additional round of shearing using the same shearing hydropore. Note that further shearing may lead to additional volume loss if a different hydropore is used.
    - While KOD Xtreme hot start polymerase can amplify DNA fragments >11 kb, amplification efficiency may be reduced when DNA is under-sheared, resulting in bi-modal HiFi read length distributions.

3. **Why do I need to optimize PCR cycles?**
    - The optimal number of PCR cycles depends on the quality and accurate quantification of the gDNA. Low-quality or low-quantity samples may require additional PCR cycles, while high-quality samples typically require fewer cycles.

4. **What are the duplication rates with this protocol?**
    - Duplication rates vary based on DNA mass input, quality, and the number of PCR cycles used. High-quality hg002 DNA gave the following duplication rates:
        - 12% with 14 PCR cycles
        - 3% with 12 cycles
        - 0.8% with 10 cycles
        - 0.3% with 8 cycles.

For lower duplication rates, we recommend using higher gDNA inputs. If using Revio SPRQ chemistry, PCR cycles may be reduced further.

5. Can I use TapeStation for sizing QC?

- TapeStation requires a higher DNA concentration than what is typically used in the Ampli-Fi protocol, so it is typically not compatible with sheared DNA QC. In addition, we have noted that sheared DNA size is inaccurate and runs significantly larger on TapeStation. However, the SMRTbell product can be QC'd with Genomic DNA ScreenTape. Note that DNA size may register as larger (up to 7kb) on the TapeStation.

Revision history (description)	Version	Date
Initial release	01	March 2025