Technical overview

Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing

Vega system ICS v1.0+
Revio system ICS v13.3+
SMRT Link v25.1+

PN 103-645-000 Rev 01 | March 2025

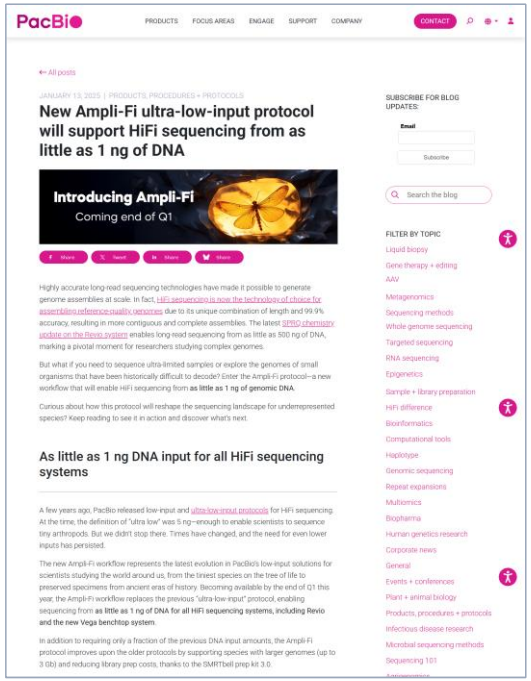
Technical overview

Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing

1. Ampli-Fi library preparation method overview
2. Ampli-Fi library preparation workflow details
3. Ampli-Fi sequencing preparation workflow details
4. Ampli-Fi data analysis recommendations for supported applications & use cases
5. Ampli-Fi example sequencing performance data
6. Technical documentation & applications support resources

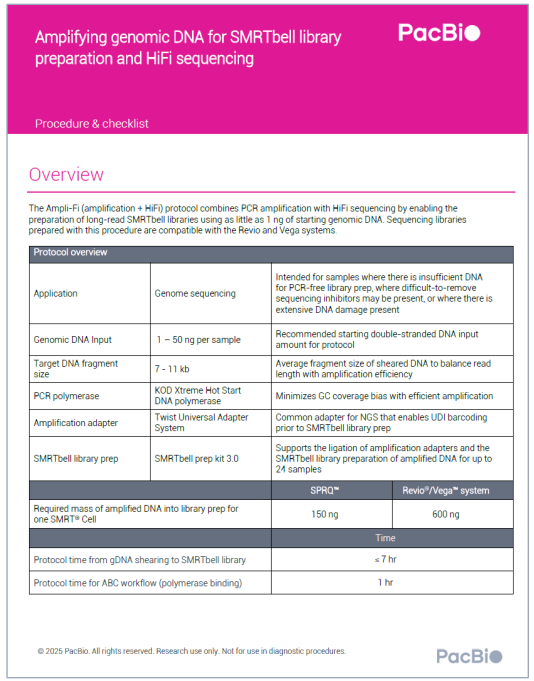
Ampli-Fi library preparation using PCR for HiFi sequencing on PacBio long-read systems: Getting started

Application-specific literature → **Application-specific protocol** → **Application-specific technical overview** → **Library preparation, sequencing & analysis**



Application notes and Application briefs [Link]

Summary overview of application-specific library preparation, sequencing and data analysis workflow recommendations.



Procedure & Checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)

Technical documentation containing library construction and sequencing preparation protocol details.

Technical overview: Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-645-000)

Technical overview presentations describe workflow details for constructing HiFi libraries for specific applications. Example sequencing performance data for a given application are also summarized.

Genomic DNA QC & shearing

1 – 50 ng of input gDNA
Shear DNA to 7 – 11 kb

DNA amplification & Library construction
(SMRTbell prep kit 3.0)

Ligate PCR amplification adapters
PCR-amplify and index DNA samples
Construct SMRTbell libraries
Optionally multiplex Ampli-Fi samples

Sequencing
(Vega & Revio systems)

Perform ABC[1] and sequence libraries on PacBio long-read systems

Data analysis
(SMRT Link or third-party tools)

E.g., genome assembly, variant detection, metagenomic analysis

[1] ABC = Anneal primer / Bind polymerase / Clean up bound complex

Ampli-Fi library preparation method overview

Ampli-Fi library preparation procedure description

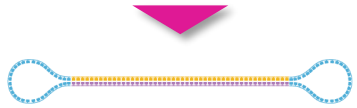
Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000) combines PCR amplification with HiFi sequencing by enabling the preparation of long-read SMRTbell libraries using as little as 1 ng of starting genomic DNA. Sequencing libraries prepared with this procedure are compatible with the Revio and Vega systems.

Protocol overview			
Application	Genome sequencing	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 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	
		SPRQ™	Revio®/Vega™ system
Required mass of amplified DNA into library prep for one 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	

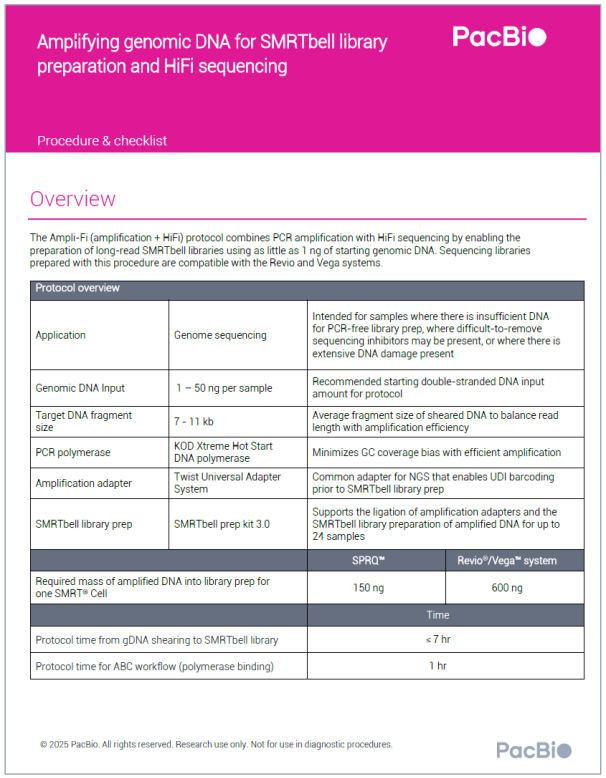
KOD Xtreme Hot Start DNA polymerase

Twist Universal Adapter System +Twist UDI primers

SMRTbell prep kit 3.0



Indexed[2] Ampli-Fi library (7 – 11 kb)



PacBio Documentation (103-648-000)

Note: For multiplexed **microbial genome assembly applications where ≥50 ng of input gDNA per sample is available**, we recommend using the HiFi plex prep kit 96 (PN: 103-381-200) and following *Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96* (103-418-800)[1]

[1] For multiplexed microbial genome assembly applications where ≥50 ng of input gDNA per sample is available, we recommend using the HiFi plex prep kit 96 (PN 103-381-200) and following ***Procedure & checklist – Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96*** (103-418-800).

[2] **Note:** This procedure (103-648-000) produces asymmetrically barcoded Ampli-Fi DNA libraries using UDI barcoding with the Twist Universal Adapter System and Twist UDI Primers, but Ampli-Fi libraries may also optionally be symmetrically barcoded using SMRTbell adapter index plate 96A (102-009-200) (or adapter index plate 96B/C/D) if desired.

SMRTbell prep kit 3.0 (SPK 3.0) Ampli-Fi library preparation workflow overview

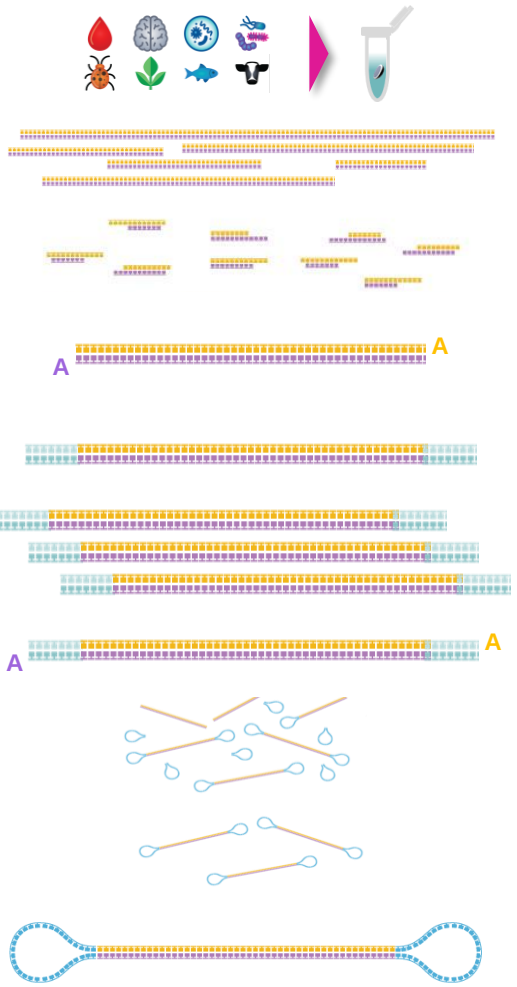
Ampli-Fi library preparation workflow

DNA shearing & amplification

- DNA sample extraction
- DNA sample QC
- DNA shearing
- DNA repair & A-tailing
- Amplification adapter ligation
- Library amplification by PCR

Library construction

- DNA repair & A-tailing
- SMRTbell adapter ligation
- Nuclease treatment
- Library size selection



Protocol documentation or reference

Refer to third-party user guide documenation

Refer to third-party user guide documenation

SPK 3.0 Ampli-Fi protocol reference

Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)

Recommended equipment & consumables

Nanobind kit *or* other 3rd-party application-specific DNA extraction kits

Qubit 4 fluorometer system
DNA sizing QC tool (e.g., Femto Pulse)

Megaruptor 3 *or* g-TUBE shearing

Twist Universal Adapter System with Twist UDI Primers

KOD Xtreme Hot Start DNA polymerase

SMRTbell prep kit 3.0

SMRTbell adapter index plate 96A
(Optional for sample multiplexing)

AMPure PB bead size selection kit[1]

[1] Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >8 kb. **Note:** For libraries with a size less than 5 kb, a 1X SMRTbell bead cleanup is recommended

SMRTbell prep kit 3.0 bundle (102-182-700)

SPK 3.0 bundle supports Ampli-Fi library preparation workflows[1]

- Contains the necessary reagents for library preparation with SMRTbell adapters
- Kit also includes SMRTbell cleanup beads and low TE buffer
- Indexed (barcoded) adapters and size-selection reagents[1] are sold separately
- Supports 24 SMRTbell libraries per kit
- Compatible with sequencing on the Vega system and Revio system

SMRTbell prep kit 3.0 bundle components

Component		Description
1		**SMRTbell prep kit 3.0** • Contains core reagents for SMRTbell template construction
2		**Low TE buffer** • For DNA shearing and cleanup
3		**SMRTbell cleanup beads** • For DNA cleanup

SMRTbell prep kit 3.0 bundle configuration

SMRTbell prep kit 3.0 (102-141-700)

#	Component	Part number	Qty	Color	Volume
1	Repair buffer	102-166-000	1	purple	220 µL
2	End repair mix	102-166-100	1	blue	110 µL
3	DNA repair mix	102-167-700	1	green	55 µL
4	SMRTbell adapter	102-167-800	1	orange	125 µL
5	Ligation mix	102-167-200	1	yellow	860 µL
6	Ligation enhancer	102-179-100	1	red	55 µL
7	Nuclease buffer	102-167-900	1	light purple	155 µL
8	Nuclease mix	102-166-200	1	light green	155 µL
9-10	Elution buffer	100-159-800	2	white	1.5 mL

Low TE buffer (102-178-400)

#	Component	Part number	Qty	Color	Volume
1	Low TE buffer (pH 8.0)	102-178-400	1	clear	10 mL

SMRTbell cleanup beads (102-158-300)

#	Component	Part number	Qty	Color	Volume
1	SMRTbell cleanup beads	102-158-300	1	clear	10 mL

[1] AMPure PB beads kit (102-182-500) for performing SMRTbell library size selection must be purchased separately.

Other recommended kits & consumables for Ampli-Fi library preparation and HiFi sequencing

Ancillary kits must be purchased separately from SMRTbell prep kit 3.0 bundle (102-182-700)

DNA extraction

Nanobind PanDNA kit
(PacBio 103-260-000)

Or

Third-party DNA extraction kits or methods

Use any suitable DNA extraction kit or method to isolate sufficient gDNA from your specific sample type of interest

DNA amplification & indexing

KOD Xtreme Hot Start DNA polymerase
(MilliporeSigma 71975-3)

Twist Universal Adapter System
(Twist Bioscience 101307-101311 / 96 rxn)

Use ultra-high fidelity DNA polymerase with Twist UDI primers[1] for PCR & unique dual indexing of long DNA fragments

Final library size selection

AMPure PB bead size selection kit
(PacBio 102-182-500)

Use AMPure PB bead size selection to progressively remove dsDNA fragments <5 kb from Ampli-Fi libraries

DNA shearing

Megaruptor 3 system
(Diagenode)

Or

g-TUBE device
(Covaris)

Shear DNA to ~7 – 11 kb target fragment size using either Megaruptor 3 system (45 min) or g-TUBE (10 min)

SMRTbell adapter indexing

OPTIONAL:
SMRTbell adapter index plate 96A
(PacBio 102-009-200)

Optionally use SMRTbell adapter index plate to symmetrically barcode Ampli-Fi libraries[1]

HiFi sequencing

Ampli-Fi procedure supports HiFi sequencing on Revio system (with/without SPRQ chemistry) & Vega system

[1] **Note:** This procedure (103-648-000) asymmetrically barcodes Ampli-Fi DNA samples using UDI barcoding with the Twist Universal Adapter System and Twist UDI Primers, but Ampli-Fi samples may also optionally be symmetrically barcoded using SMRTbell adapter index plate 96A (102-009-200) (or adapter index plate 96B/C/D) if desired.

Ampli-Fi application supported use cases and sequencing performance considerations

Example Ampli-Fi sequencing use cases and applications

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

Sample type	Example
❑ Samples that do not have sufficient DNA mass for PCR-free sequencing	Single, small insects
❑ Samples contaminated with sequencing inhibitors that are challenging to remove	Snail or marine organisms
❑ Samples derived from preserved specimens collected in the field	Ethanol-preserved tissue
❑ Samples derived from formalin-fixed paraffin-embedded (FFPE) tissues	FFPE tumor samples
❑ Samples derived from chromosome conformation capture (3C) experiments	CiFi application[1] samples

Ampli-Fi sequencing performance considerations

- Sequencing yield expectations should be set in accordance with input gDNA quality
- Samples containing higher amounts of degraded or fragmented DNA will produce lower average library insert sizes due to preferential amplification of shorter DNA fragments during the PCR step
  - → Shorter library inserts will lead to reduced HiFi read lengths and lower HiFi data yields
- To maximize HiFi base yield and average read length, it is recommended to start with high-molecular weight (HMW) gDNA whenever possible

PacBio system	Ampli-Fi HiFi read length	Ampli-Fi HiFi yield per SMRT Cell
Revio system (SPRQ chemistry)	~5 – 10 kb	~35 – 70 Gb
Vega system	~5 – 10 kb	~25 – 50 Gb

[1] CiFi method couples chromatin conformation capture (3C) with PacBio HiFi long-read sequencing using low DNA input amounts (see McGinty et al. CiFi: Accurate long-read chromatin conformation capture with low-input requirements. BioRxiv preprint DOI: https://doi.org/10.1101/2025.01.31.635566).

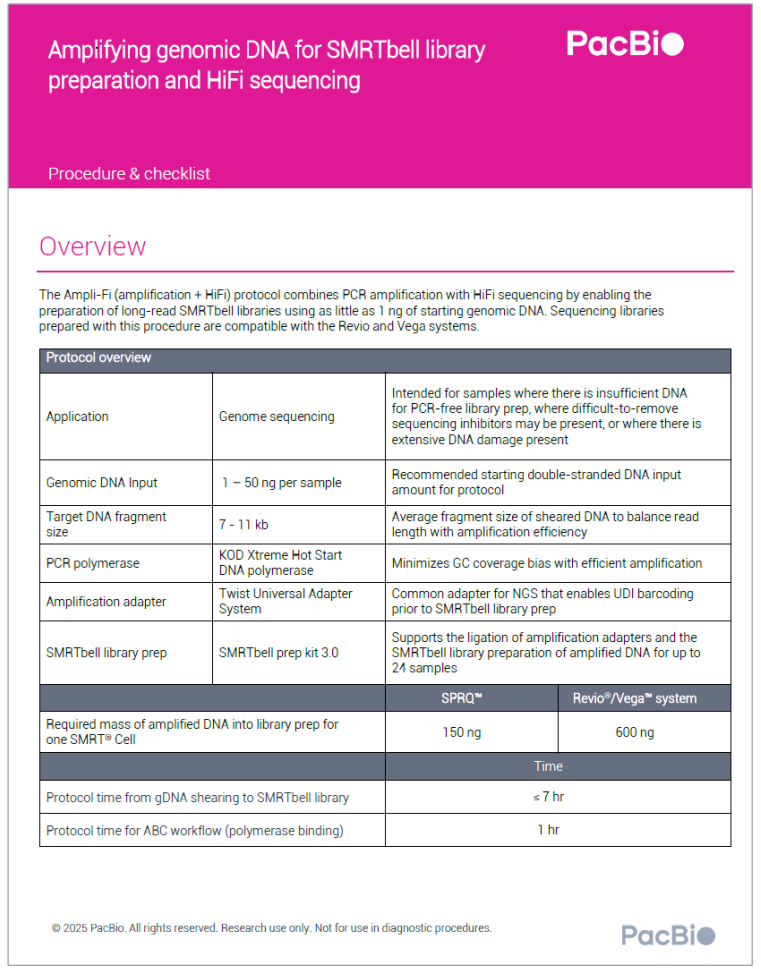
Ampli-Fi library preparation workflow details

Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)

Procedure & checklist 103-648-000 combines PCR amplification with HiFi sequencing by enabling the preparation of long-read SMRTbell libraries using as little as 1 ng of starting genomic DNA. Sequencing libraries prepared with this procedure are compatible with the Revio and Vega systems.

Procedure & checklist contents

1. Genomic DNA (gDNA) input QC recommendations and general best practices for reagent & sample handling.
2. Instructions for DNA shearing using Megaruptor 3 system (Diagenode) or g-TUBEs (Covaris).
3. Instructions for performing PCR amplification & indexing of DNA samples using Twist Universal Adapter System with Twist UDI primers (Twist Bioscience) and KOD Xtreme Hot Start DNA polymerase.
4. Enzymatic workflow steps for SMRTbell library construction using SMRTbell prep kit 3.0.
5. Instructions for performing final cleanup and size selection on SMRTbell library using AMPure PB beads.
6. Workflow steps for sample setup ABC[1] (annealing, binding, and cleanup) to prepare samples for sequencing using Vega polymerase kit or Revio SPRQ polymerase kit.



Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing

PacBio

Procedure & checklist

Overview

The Ampli-Fi (amplification + HiFi) protocol combines PCR amplification with HiFi sequencing by enabling the preparation of long-read SMRTbell libraries using as little as 1 ng of starting genomic DNA. Sequencing libraries prepared with this procedure are compatible with the Revio and Vega systems.

Protocol overview		
Application	Genome sequencing	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 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

	SPRQ™	Revio®/Vega™ system
Required mass of amplified DNA into library prep for one 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	

PacBio

PacBio Documentation (103-648-000)

[1] For primer annealing, polymerase binding & complex cleanup (ABC) using Vega chemistry or Revio SPRQ chemistry, follow sample setup instructions in Procedure & checklist – Do not use SMRT Link Sample Setup. For ABC using Revio non-SPRQ chemistry, follow SMRT Link Sample Setup ABC Calculator software for preparing samples for sequencing.

Key Ampli-Fi library preparation and sequencing workflow steps

Ampli-Fi library preparation + ABC workflow can be completed within 1 day[1]

QC

DNA sample extraction & QC — 2 – 2.5 hrs

Nanobind *or* 3rd-party application-specific DNA extraction kit
Qubit 4 fluorometer system (ThermoFisher Sci. Q33238)
DNA sizing QC tool (Femto pulse system or other tool)

Refer to third-party user guide documenation

DNA Shearing & amplification

1 Shear DNA — 10 min

Megaruptor 3 (Diagenode B06010003) *or*
g-TUBE (Covaris 520104)

2 Repair & A-tailing of sheared DNA — 45 min

3 Ligation of amplification adapter and cleanup — 1 hr

Twist Universal Adapter System with Twist UDI Primers (Twist Bioscience 101307-101311) (for PCR amplification and sample unique dual indexing)

Safe stop

4 Library amplification by PCR and cleanup — 2.5 hrs

KOD Xtreme Hot Start DNA polymerase (Millipore Sigma 71975-3) (for PCR amplification)

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA — 45 min

SMRTbell prep kit 3.0
(102-182-700) (for SMRTbell library construction)

6 SMRTbell adapter ligation and cleanup — 1 hr

SMRTbell adapter index plate 96A
(102-009-200) (Optional for sample symmetric indexing)

Safe stop

7 Nuclease treatment — 20 min

8 AMPure PB bead size selection — 30 min

AMPure PB bead size selection kit (102-182-500)[2]

ABC

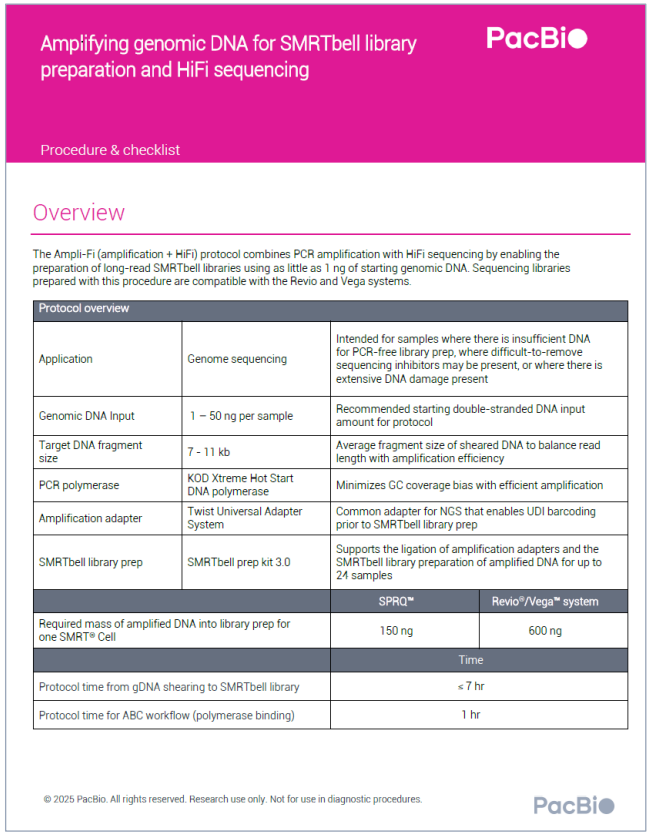
9 Annealing, binding & cleanup (ABC) — 1 hr

Revio SPRQ polymerase kit (103-496-900) *or*
Revio polymerase kit (non-SPRQ) (102-739-100) *or*
Vega polymerase kit (103-426-500)

1 DAY

Ampli-Fi protocol reference

Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)



Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing

PacBio

Procedure & checklist

Overview

The Ampli-Fi (amplification + HiFi) protocol combines PCR amplification with HiFi sequencing by enabling the preparation of long-read SMRTbell libraries using as little as 1 ng of starting genomic DNA. Sequencing libraries prepared with this procedure are compatible with the Revio and Vega systems.

Protocol overview		
Application	Genome sequencing	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 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
	SPRQ™	Revio®/Vega™ system
Required mass of amplified DNA into library prep for one 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	

PacBio

PacBio

[1] Example workflow step times shown are for manually processing up to 8 Ampli-Fi samples using a g-TUBE device for DNA shearing and performing library amplification using 10 PCR cycles.
[2] Can optionally perform gel cassette-based size selection to enrich for SMRTbell library inserts >8 kb.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0

DNA sample extraction

Example DNA extraction kits for challenging sample types

Note: Third-party products listed below have not been extensively tested or validated by PacBio but are listed here as examples of kits or methods used by other PacBio customers for isolating genomic DNA from challenging sample types

Sample type	Third-party product or kit
Formalin-fixed paraffin-embedded (FFPE) samples	QIAGEN QIAamp DNA FFPE Tissue Kit (PN 56404)
Dried blood spots	QIAGEN QIAamp DNA Blood Mini Kit (PN 51104)
Fecal and soil	QIAGEN DNeasy PowerSoil Pro (PN 47014)
	QIAGEN PowerFecal Pro (PN 51804)
	QIAGEN DNeasy PowerClean Pro Cleanup Kit (PN 12997-50) • If needed, can be used after extracting DNA with PowerSoil or PowerFecal kits to further improve sequencing performance

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

DNA sizing QC

- If available, Agilent Femto Pulse system[1] is recommended for the accurate sizing of DNA samples
  - Femto Pulse system employs pulsed-field capillary electrophoresis technology and enables simple, rapid sizing QC of genomic DNA and SMRTbell libraries, and conserves sample by using femtogram ranges of input DNA
    - Resolves fragments 1.3 kb to 165 kb using gDNA 165 kb Analysis kit (can resolve 100 – 6,000 bp using Ultra Sensitivity NGS kit)
    - Requires <1 ng of sample DNA
    - Can analyze up to 12 samples in <1.5 hrs
    - Outputs quality metrics such as Genomic Quality Number (GQN)[2] to quickly score integrity of HMW gDNA
- Alternative DNA sizing tools may be used if a Femto Pulse system is unavailable
  - However, caution should be used when interpreting results from other tools that employ constant-field electrophoresis technology
    - These technologies tend to inflate the true size of the gDNA (or library) and should only be used for qualitative assessment of whether an experiment was successful (e.g., intact library) rather than for accurate measurement of fragment size distributions

Femto Pulse system (Agilent Technologies)

DNA quantification QC

- For DNA quantification QC, we recommend using a quantification assay specific for double-stranded DNA (dsDNA) such as the Qubit 1X dsDNA high sensitivity assay kit[3] (Thermo Fisher Scientific)
  - **Note:** We do not recommend quantification with UV-Vis Spectrophotometers (e.g. NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples

Qubit 4 fluorometer (Thermo Fisher Scientific)

[1] See ***Product Note – HiFi WGS sequencing with the Agilent Femto Pulse system*** (102-326-561) for more details.

[2] See ***Application Note – Quality Metrics for Nucleic Acids with the Agilent Fragment Analyzer and Femto Pulse Systems*** (Agilent 5994-0521EN)

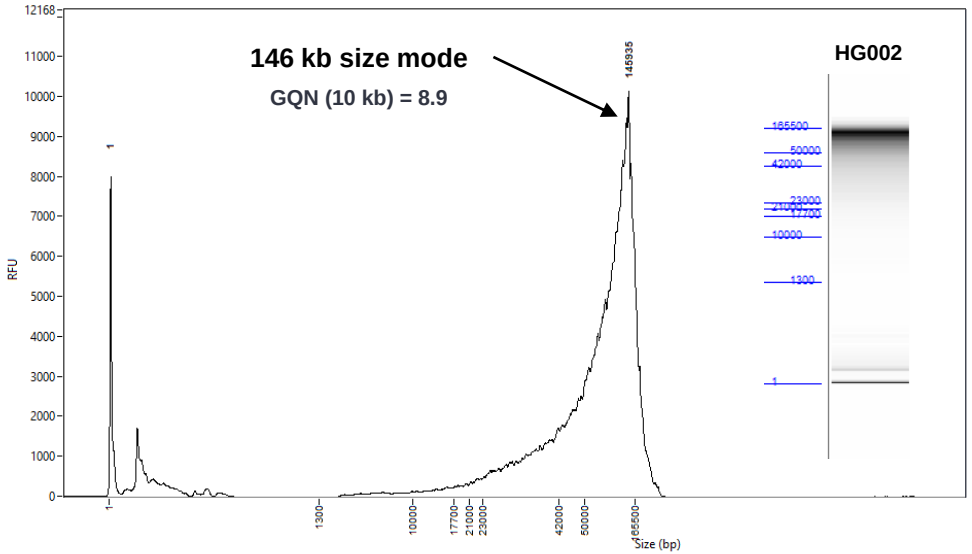
[3] Alternatively, for high-throughput applications DNA quantification QC may be performed with a microplate reader using the Quant-iT 1X dsDNA high sensitivity assay kit (Thermo Fisher Scientific).

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Recommended DNA input amount and quality

Genomic DNA input quality

- For Ampli-Fi applications, using higher quality input DNA will produce improved overall HiFi sequencing data quality[1,2]
  - Where possible, we recommend using input gDNA with a genome quality number (GQN) of 7.0 or higher at 10 kb ($GQN_{10kb} \geq 7.0$)
  - Lower quality input DNA ($GQN_{10kb} < 7.0$; e.g., FFPE samples) may be used; however, shorter DNA fragments (< 10 kb) will tend to be preferentially amplified and thus lead to lower mean HiFi read lengths and reduced HiFi sequencing data yields



Any degradation present should be due to shearing from extraction process and **not** from poor sample handling/storage or biochemical processes

Example DNA sizing QC analysis of a high-quality HG002 human genomic DNA sample using a Femto Pulse system with Genomic DNA 165 kb kit.

[1] **Important:** The HiFi yield and HiFi mean read length of a sequencing run are directly proportional to the quality of the genomic DNA input and the fragment lengths generated after shearing. To maximize yield and genome coverage per SMRT Cell, start with high quality gDNA containing minimal DNA below 10 kb. High quality gDNA will typically have a higher percent library recovery and HiFi sequencing yield.

[2] Gel size selection approaches can be used with the Ampli-Fi protocol to improve HiFi read length for certain samples that have a relatively large fraction of short DNA (< 5 kb). To remove unwanted DNA fragments larger than 5 kb using alternative gel-based size selection methods, please see ***Technical note – Gel cassette size selection methods for WGS HiFi libraries*** (102-326-503).

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Recommended DNA input amount and quality (cont.)

Genomic DNA input amount required for shearing[1]

Starting gDNA input into shearing	Vega system	Revio system with non-SPRQ chemistry	Revio system With SPRQ chemistry
Recommended starting genomic dsDNA input amount for protocol.	1 – 50 ng per sample		

Amplified DNA input amount required for SMRTbell library preparation using SMRTbell prep kit 3.0

Amplified DNA input into SPK 3.0 library prep	Vega system	Revio system with non-SPRQ chemistry	Revio system With SPRQ chemistry
Amount of amplified DNA required for one SMRT Cell[1]	600 ng per SMRT Cell	600 ng per SMRT Cell	150 ng per SMRT Cell

PacBio

[1] A mean sheared DNA fragment size between 7 to 11 kb with a narrow distribution (typically ~5 – 20 kb) is recommended for library construction using this protocol → If the starting gDNA input is within these ranges or lower, then the DNA shearing step can be bypassed.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Ampli-Fi library construction yields

Expected SPK 3.0 Ampli-Fi library construction yield

- Overall SMRTbell library construction yield is dependent on input amplified DNA quality and size
  - The recovery from input amplified DNA to polymerase-bound SMRTbell library typically ranges between ~20 – 40% (includes SMRTbell library construction and ABC)

Protocol step	Expected DNA or SMRTbell step recovery	Expected DNA or SMRTbell overall recovery	Example DNA or SMRTbell overall recovery	Expected size (Femto Pulse)
Starting amplified DNA input	100%	100%	600 ng	7 – 11 kb
Post-SMRTbell adapter ligation & SMRTbell bead cleanup	80 – 95%	80 – 95%	480 – 570 ng	7 – 11 kb
Post-nuclease (pre-cleanup)	40 – 50%	32 – 48%	192 – 288 ng	7 – 11 kb
Post-3.1x AMPure PB bead cleanup	75 – 80%	24 – 38%	144 – 228 ng	7 – 11 kb
Post-ABC cleanup	75 – 95%	18 – 36%	108 – 216 ng	7 – 11 kb

Minimum polymerase-bound Ampli-Fi library mass needed to load a SMRT Cell

Mean library insert size	Polymerase-bound Ampli-Fi library mass needed to load one SMRT Cell (120 pM OPLC)[1]		
	Vega system	Revio system with non-SPRQ chemistry	Revio system With SPRQ chemistry
10,000 bp	100 ng	100 ng	25 ng

[1] Recommended on-plate loading concentration (OPLC) range for Ampli-Fi libraries (~5-10 kb) is ~120 – 160 pM for Revio system and ~100 – 140 pM for Vega system. **Note:** Starting with 600 ng of amplified DNA (going into SMRTbell library prep with SPK 3.0) will, on average, provide enough (~5-10 kb) library to load 2 Revio SMRT Cells without SPRQ chemistry or 2 Vega SMRT Cells. Starting with 150 ng of amplified DNA (going into SMRTbell library prep with SPK 3.0) will, on average, provide enough (~5-10 kb) library to load ≥2 Revio SMRT Cells with SPRQ chemistry.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

DNA shearing

- A mean fragment size between 7 to 11 kb with a narrow distribution (typically ~5 – 20 kb) is recommended for this protocol
  → **If the starting genomic DNA is within these ranges or lower, the DNA shearing step can be bypassed**
- We recommend performing DNA shearing using a Megaruptor 3 system (Diagenode) or g-TUBEs (Covaris)

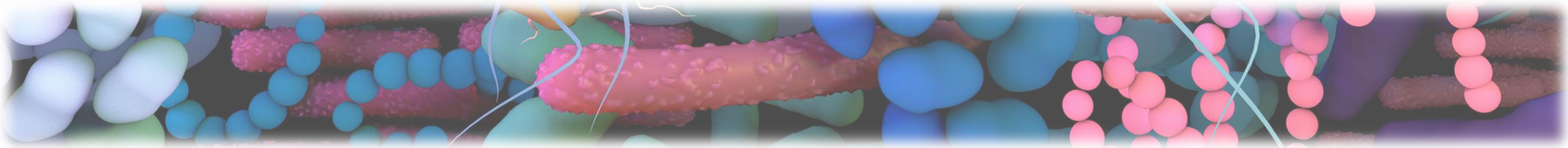
	Megaruptor 3 system	g-TUBE
Input DNA mass	1 – 50 ng	1 – 50 ng
Shearing volume	65 µL	65 µL
Target insert length (mode)	10 kb	10 kb
Shearing conditions	Shear speed = 55 – 59	2348 x *g* (5000 rpm in Eppendorf 5424R) for 5 minutes; total # of passes = 2

Megaruptor 3 (Diagenode)

g-TUBE (Covaris)

Metagenomic samples

- Metagenomic samples often have degraded gDNA where the majority of fragments are already <15 kb in length to start
  → If DNA sizing QC indicates that the average fragment size of the starting gDNA is <15 kb, then skip the DNA shearing step in this procedure



General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling – PacBio kits or consumables[1]

PacBio kit or consumable	Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice	Bring these reagents to room temperature 30 minutes prior to use
SMRTbell prep kit 3.0 (102-182-700) 	❑ Repair buffer	❑ End repair mix	❑ Elution buffer
	❑ Nuclease buffer	❑ DNA repair mix	❑ SMRTbell cleanup beads
	❑ SMRTbell adapter	❑ Ligation mix	
	❑ Elution buffer	❑ Ligation enhancer	
		❑ Nuclease mix	
		❑ SMRTbell adapter	
AMPure PB bead size selection kit (102-182-500) 	❑ Elution buffer	❑ N/A	❑ Elution buffer
			❑ AMPure PB beads

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling – PacBio kits or consumables[1] (cont.)

PacBio reagent handling notes

- Room temperature is defined as any temperature in the range of 18 – 25°C for this protocol
- Once thawed, reaction buffers & adapter index plate may be stored on cold block, at 4°C, or on-ice prior to making master mix or placing on liquid handler work deck
- Mix reagent buffers with a brief vortex prior to use (do not vortex enzymes)
- Briefly spin down all reagent tubes in a microcentrifuge to collect all liquid at the bottom (if using a SMRTbell adapter index plate, briefly vortex and then spin down in a centrifuge with a plate adapter to collect all liquid at the bottom of the wells)
- Vortex SMRTbell cleanup beads and AMPure PB beads immediately before use (failure to do this will result in low recovery)
- Pipette-mix (e.g., up and down 10 times) all bead binding and elution steps until beads are distributed evenly in solution
- Samples can be stored at 4°C at all safe stopping points listed in the protocol

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Reagent handling – Third-party kits or consumables[1]

Third-party kit or consumable	Thaw these reagents at room temperature	Keep these temperature-sensitive reagents on ice	Bring these reagents to room temperature 30 minutes prior to use
Twist Universal Adapter System (Twist Bioscience 101307-101311) 	❑ Twist universal adapter and UDI primers	❑ Twist universal adapter and UDI primers	❑ N/A
KOD Xtreme Hot Start DNA polymerase (Millipore Sigma 71975-3) 	❑ 2x Xtreme buffer	❑ 2x Xtreme buffer	❑ N/A
	❑ dNTP	❑ dNTP	
Qubit dsDNA HS assay kit (ThermoFisher Scientific Q33230) 	❑ N/A	❑ N/A	❑ dsDNA quantification reagents

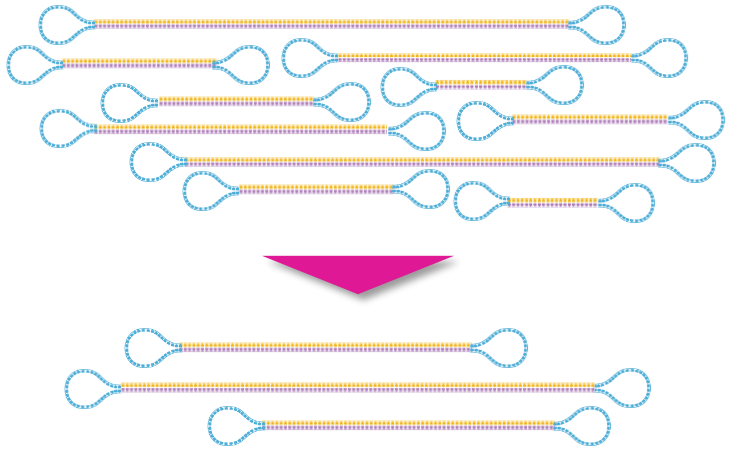
[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required library preparation reagents.

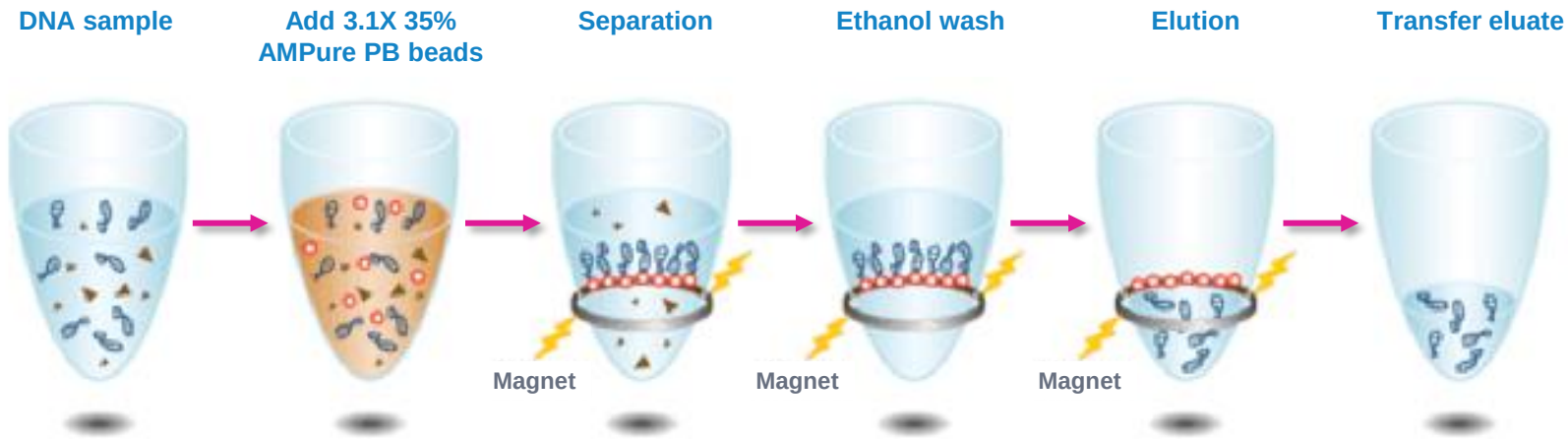
General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Final library size selection using AMPure PB bead size selection kit (102-182-500)

- AMPure PB beads are used as the default size selection method to progressively deplete short DNA fragments <5 kb[1] from final SPK 3.0 Ampli-Fi libraries and enrich for long fragments

AMPure PB bead size selection kit
(102-182-500)





AMPure PB bead size selection procedure

1. Prepare a **35% dilution (v/v)** of the AMPure PB bead stock in Elution Buffer (EB)
   - 35% AMPure PB beads solution can be stored at 4°C for 30 days.
2. Add 3.1X of room-temperature 35% AMPure PB beads to each sample and incubate for 20 min at RT
3. Place samples on magnetic rack; wash samples with 80% ethanol 2X; then elute samples in EB for 5 min at RT

[1] Gel size selection approaches can be used with the Ampli-Fi protocol to improve HiFi read length for certain samples that have a relatively large fraction of short DNA (< 5 kb). To remove unwanted DNA fragments larger than 5 kb using alternative gel-based size selection methods, please see ***Technical note – Gel cassette size selection methods for WGS HiFi libraries*** (102-326-503).

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Multiplexing samples

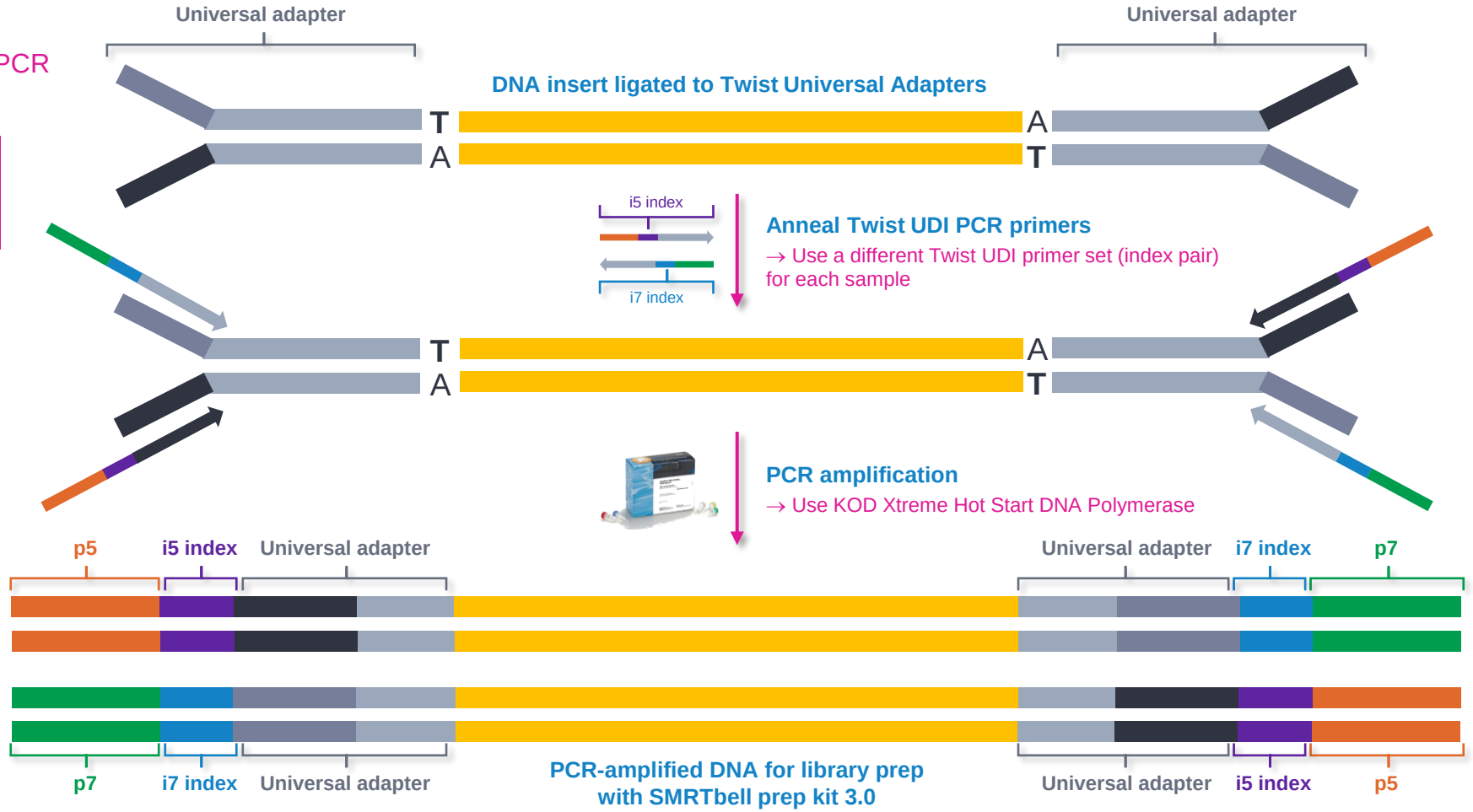
Sample indexing (barcoding) using Twist Universal Adapters and Twist Unique Dual Indexed (UDI) Primers

- In this procedure, **all** Ampli-Fi samples are indexed (asymmetrically barcoded) during PCR amplification step using Twist UDI primers

Twist UDI primers provide **unique dual-indexed** combinations (10 bp i5 index + 10 bp i7 index) with 1 PCR reaction per index pair

- Twist UDI primers are configured in 96-well plates and are available in 16-or 96-sample formats[1]
- To perform sample demultiplexing, use the following barcode set[2] that includes the appropriate Twist Universal Adapter and Twist UDI primer sequences:
  - Amplifi_TwistUDIadapters_noP7P5 [Link]

```
UDI<------- Universal adapter ----->
[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT

UDI<------- Universal adapter ----->
[i7]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
```



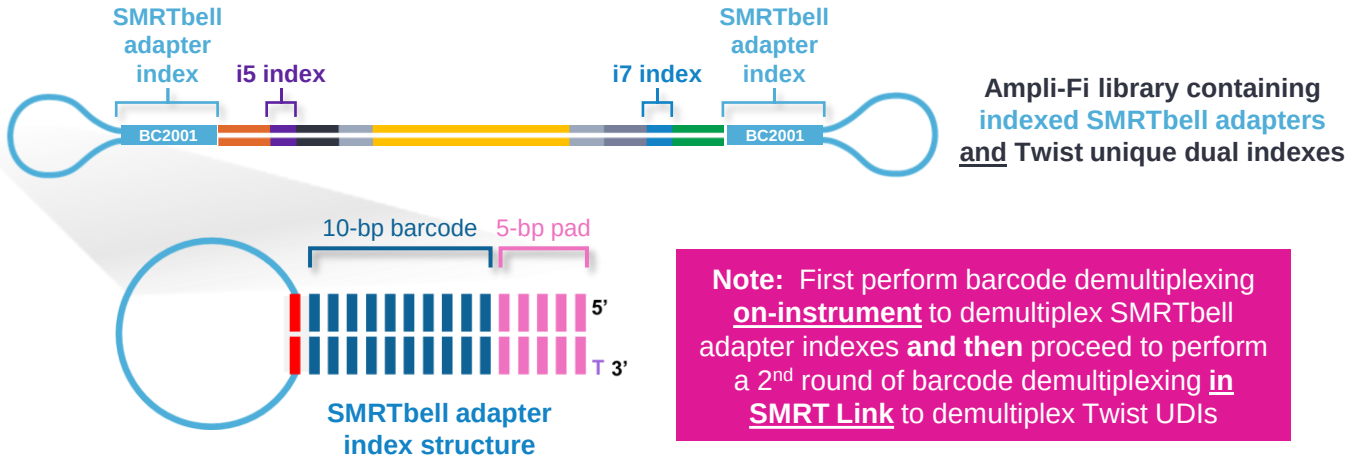
[1] In the 96-sample format, 4 unique plates are available to allow for multiplexing 384 samples in a single sequencing run. Refer to Twist Bioscience website for more information about Twist UDI sequences.
[2] Refer to data analysis workflow recommendations in Section 4 of this technical overview presentation for more information about appropriate barcode sets to use for Ampli-Fi sample demultiplexing.

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Multiplexing samples (cont.)

OPTIONAL: Sample indexing (barcoding) using SMRTbell adapter index plate 96A/B/C/D

- If multiplexing Ampli-Fi samples, can **optionally** use SMRTbell adapter index plate 96A/B/C/D to index (symmetrically barcode) your libraries during SMRTbell adapter ligation step[1]
  - **Note:** In the standard Ampli-Fi library preparation workflow (103-648-000), **all** DNA samples are asymmetrically barcoded using UDI barcoding with the Twist Universal Adapter System and Twist UDI Primers → we recommend using this method (instead of using SMRTbell adapter index plate) for multiplexing Ampli-Fi libraries



Ampli-Fi library containing indexed SMRTbell adapters and Twist unique dual indexes

Note: First perform barcode demultiplexing **on-instrument** to demultiplex SMRTbell adapter indexes **and then** proceed to perform a 2nd round of barcode demultiplexing **in SMRT Link** to demultiplex Twist UDIs

- SMRT Link comes preloaded with the following barcode set FASTA file containing SMRTbell adapter index plate 96A/B/C/D barcode sequences:
  - ❑ `SMRTbell adapters indexes` (for Revio & Vega system run designs)

	1	2	3	4	5	6	7	8	9	10	11	12
A	BC2001	BC2009	BC2017	BC2025	BC2033	BC2041	BC2049	BC2057	BC2065	BC2073	BC2081	BC2089
B	BC2002	BC2010	BC2018	BC2026	BC2034	BC2042	BC2050	BC2058	BC2066	BC2074	BC2082	BC2090
C	BC2003	BC2011	BC2019	BC2027	BC2035	BC2043	BC2051	BC2059	BC2067	BC2075	BC2083	BC2091
D	BC2004	BC2012	BC2020	BC2028	BC2036	BC2044	BC2052	BC2060	BC2068	BC2076	BC2084	BC2092
E	BC2005	BC2013	BC2021	BC2029	BC2037	BC2045	BC2053	BC2061	BC2069	BC2077	BC2085	BC2093
F	BC2006	BC2014	BC2022	BC2030	BC2038	BC2046	BC2054	BC2062	BC2070	BC2078	BC2086	BC2094
G	BC2007	BC2015	BC2023	BC2031	BC2039	BC2047	BC2055	BC2063	BC2071	BC2079	BC2087	BC2095
H	BC2008	BC2016	BC2024	BC2032	BC2040	BC2048	BC2056	BC2064	BC2072	BC2080	BC2088	BC2096

SMRTbell adapter index plate 96A (102-009-200) contains 96 barcoded adapters to support multiplexed SMRTbell library construction for up to 96 samples using SPK 3.0

SMRTbell adapter index sequences (FASTA) [Link]

Product insert – SMRTbell adapter index plate 96A (contains plate map [Link]

[1] If barcoding Ampli-Fi samples using SMRTbell adapter index plate 96A/B/C/D, then **first** perform barcode demultiplexing **on-instrument** to demultiplex adapter-barcoded samples **and then** proceed to run Demultiplex Barcodes **in SMRT Link** to trim Twist universal adapters and demultiplex Twist UDIs (refer to Ampli-Fi data analysis workflow recommendations described in Section 4 of this presentation).

General best practices recommendations for preparing Ampli-Fi libraries using SMRTbell prep kit 3.0 (cont.)

Sequencing preparation (ABC) and polymerase-bound library storage

- ***Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing*** (103-648-000) includes instructions for the primer annealing, polymerase binding & complex cleanup (ABC) sample setup steps for Revio and Vega systems
  - → For sequencing Ampli-Fi SMRTbell libraries on the Revio system with SPRQ chemistry or the Vega system: Follow sample setup instructions stated in the protocol to perform ABC and final loading dilution procedure – Do not use SMRT Link Sample Setup software
- Sequencing polymerase is stable once bound to the SMRTbell library and can be stored at 4°C or frozen at -20°C.
- Stored polymerase-bound library shows equivalent loading to freshly prepared bound libraries up to the recommendations listed below.

  Recommended polymerase-bound storage:[1]
  - Polymerase-bound libraries can be stored at 4°C for up to 1 month
  - Polymerase-bound libraries can be stored at -20°C for up to 6 months
  - Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles
- Stored polymerase-bound libraries should be protected from light since Loading buffer is light-sensitive

[1] Polymerase-bound storage recommendations described here apply to SMRTbell libraries bound to either Vega polymerase, Revio polymerase or Revio SPRQ polymerase.

DNA sample extraction

Use any suitable DNA extraction kit or method to isolate sufficient gDNA from your specific sample type of interest

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

Example DNA extraction kits for challenging sample types

Sample type	Third-party product or kit
FFPE	QIAGEN QIAamp DNA FFPE Tissue Kit (PN 56404)
Dried blood spots	QIAGEN QIAamp DNA Blood Mini Kit (PN 51104)
Fecal and soil	QIAGEN DNeasy PowerSoil Pro (PN 47014)
	QIAGEN PowerFecal Pro (PN 51804)
	QIAGEN DNeasy PowerClean Pro Cleanup Kit (PN 12997-50) • If needed, can be used after extracting DNA with PowerSoil or PowerFecal kits to further improve sequencing performance

Note: Third-party products listed in table have not been extensively tested or validated by PacBio but are listed here as examples of kits or methods used by other PacBio customers for isolating genomic DNA from challenging sample types.

- **For FFPE samples:** We recommend using the **QIAamp DNA FFPE Tissue Kit** for DNA extraction and following the **Qiagen Supplementary Protocol**[1], which uses Deparaffinization Solution (PN 19093)

[1] **QIAGEN supplementary protocol:** *Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution (EN)* [Link]

DNA sample QC

Perform DNA QC using a Qubit dsDNA HS assay and a DNA sizing tool

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

DNA quantification QC

Qubit 4 fluorometer (Thermo Fisher Scientific)

Qubit fluorometer in conjunction with Qubit 1X dsDNA high-sensitivity assay (Thermo Fisher Scientific) enables rapid, specific and accurate determination of nucleic acid concentrations in a single sample[1,2]

- Assay is highly selective for dsDNA over ssDNA, RNA, protein, and free nucleotides. Contaminants, such as salts, solvents, or detergents are well-tolerated.
- Depending on sample volume, assay kit is designed to be accurate for initial DNA sample concentrations of 5 pg/µL to 120 ng/µL, providing a detection range of 0.1−120 ng.

DNA sizing QC

Femto Pulse system (Agilent Technologies) or other DNA sizing QC tool

Femto Pulse system enables simple, rapid sizing QC of genomic DNA and SMRTbell libraries, and conserves sample by using femtogram ranges of input DNA

- Use the Femto Pulse gDNA 165 kb analysis kit (FP-1002-0275)
- Dilute samples to 250 pg/uL

If a Femto Pulse system is unavailable, can consider using alternative DNA sizing QC systems

- Caution should be used when interpreting results from other tools that employ constant-field electrophoresis technology[3]

[3] These technologies tend to inflate the true size of the gDNA (or library) and should only be used for qualitative assessment of whether an experiment was successful (e.g., construction of intact SMRTbell library) rather than for accurate measurement of fragment size distributions

[1] **Important: The Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer (concentration readings will not be accurate).**.

[2] **Note:** We do not recommend quantification with UV-Vis Spectrophotometers (e.g., NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples.

DNA sample QC

Perform DNA QC using a Qubit dsDNA HS assay and a DNA sizing tool

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

DNA quantification QC

Qubit 4 fluorometer (Thermo Fisher Scientific)

Qubit fluorometer in conjunction with Qubit 1X dsDNA high-sensitivity assay (Thermo Fisher Scientific) enables rapid, specific and accurate determination of nucleic acid concentrations in a single sample[1,2]

- Assay is highly selective for dsDNA over ssDNA, RNA, protein, and free nucleotides. Contaminants, such as salts, solvents, or detergents are well-tolerated.
- Depending on sample volume, assay kit is designed to be accurate for initial DNA sample concentrations of 5 pg/µL to 120 ng/µL, providing a detection range of 0.1−120 ng.

DNA sizing QC

Example comparison of Ampli-Fi library sizing results: Femto Pulse system *vs.* TapeStation system

TapeStation sizing measurements are often several kb larger than Femto Pulse sizing results for ~10 kb Ampli-Fi library size range

TapeStation system

Femto Pulse system

	Measured Ampli-Fi SMRTbell library mode size (bp)	
DNA input for Ampli-Fi library prep	**TapeStation[3]**	**Femto Pulse[4]**
1 ng	14302	11799
5 ng	13024	11294
20 ng	11953	11013
50 ng	14043	10282

[3] TapeStation measurements were performed using Genomic DNA ScreenTape.
[4] Femto Pulse measurements were performed using Genomic DNA 165 kb Kit.

[1] **Important: The Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer (concentration readings will not be accurate).**.
[2] **Note:** We do not recommend quantification with UV-Vis Spectrophotometers (e.g., NanoDrop) that measure all nucleic acids in a sample. For example, measuring all nucleic acid will inflate the true concentration of gDNA in samples.

DNA shearing

Perform DNA shearing for Ampli-Fi samples using Megaruptor 3 system or g-TUBEs[1]

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

1. DNA shearing options

Option 1: DNA shearing with the Megaruptor 3 system

✔	Step	Instructions DNA shearing and cleanup
		DNA shearing
	1.1	Bring 1 -50 ng DNA up to a final volume of **65** µL with low TE buffer.
		Shear DNA on the Megaruptor 3 system with the following parameters:

Shear speed	Target insert length
59	10 kb

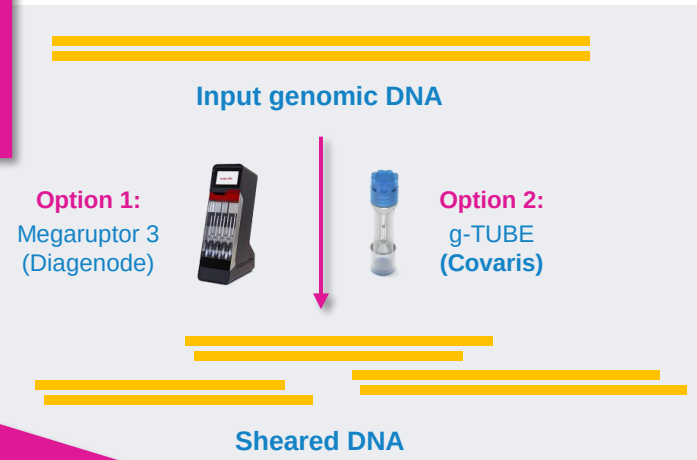
	1.2	**Note:** It is recommended to confirm gDNA is sheared to the appropriate size-range (<11 kb) prior to proceeding. If the DNA is under-sheared, a second shear with the same parameters can be repeated. The same Megaruptor consumables can be used if a secondary shear is required.
	1.3	Transfer sheared 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 >11kb, repeat step 1.2 (see note).

Option 2: Shearing genomic DNA using a Covaris g-TUBE

✔	Step	Instructions for Covaris g-TUBE shearing
	1.1	Dilute 1 -50 ng 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

- For both MR3 & g-TUBE shearing, bring 1-50 ng DNA up to a final volume of **65 µL** with low TE buffer



- If the DNA is under-sheared, a second MR3 shear with the same parameters and same consumable can be repeated

IMPORTANT!

- A mean fragment size between 7 to 11 kb is recommended for this protocol
- In addition, the distribution of fragment sizes should be narrow and generally between ~5 to 20 kb
  - Fragments that are too short produce less HiFi data per read, and fragments that are too long may not be efficiently amplified during PCR step (Step 4)

- **Note:** Can proceed with **up to 60 µL** of post-sheared DNA sample into Repair & A-tailing step (Step 2)

PacBio

Repair & A-tailing of sheared DNA

Repair sites of DNA damage and prepare sheared DNA for ligation to PCR amplification adapter

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

2. Repair and A-tailing of sheared DNA

✔	Step	Instructions for DNA damage and end repair of sheared DNA

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.

2.1

Repair mastermix

✔	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 mastermix calculations

2.2 Pipette-mix the Repair mastermix.

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

2.4 Add 11 µL of the Repair mastermix 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.

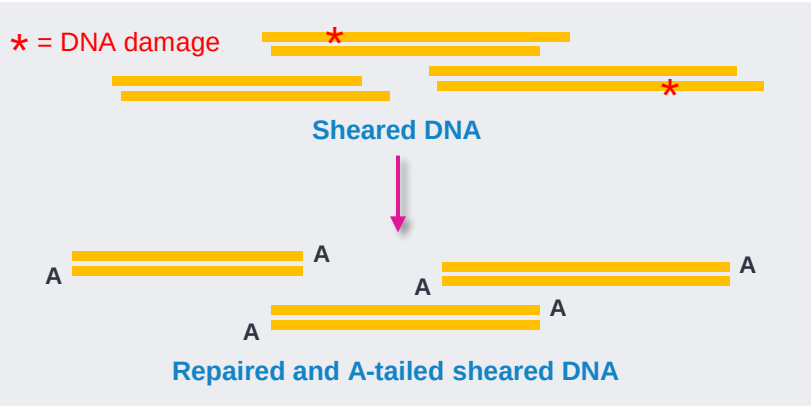
2.5 Pipette-mix each sample.

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.



- Prepare a reaction master mix by adding the required components in the order and volume listed to a new microcentrifuge tube
- Adjust component volumes for the number of samples being prepared (e.g., 4, 8 or more[1]), plus 15% overage

- Add **11 µL** of master mix to each sample
- Can use **up to 60 µL** of sheared DNA sample in Repair & A-tailing reaction → **Total rxn vol. may be up to 71 µL**

- Run **Repair and A-tailing** thermal cycler program
- Set lid temperature to **≥75°C** if programmable

[1] SMRTbell prep kit 3.0 contains a sufficient volume of reagents to support 24 library prep reactions.

Ligation of amplification adapter and cleanup

Ligate PCR amplification adapter to the ends of each DNA fragment

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

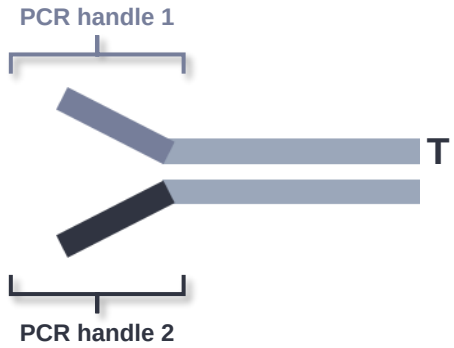
6 SMRTbell adapter ligation and cleanup

Safe stop

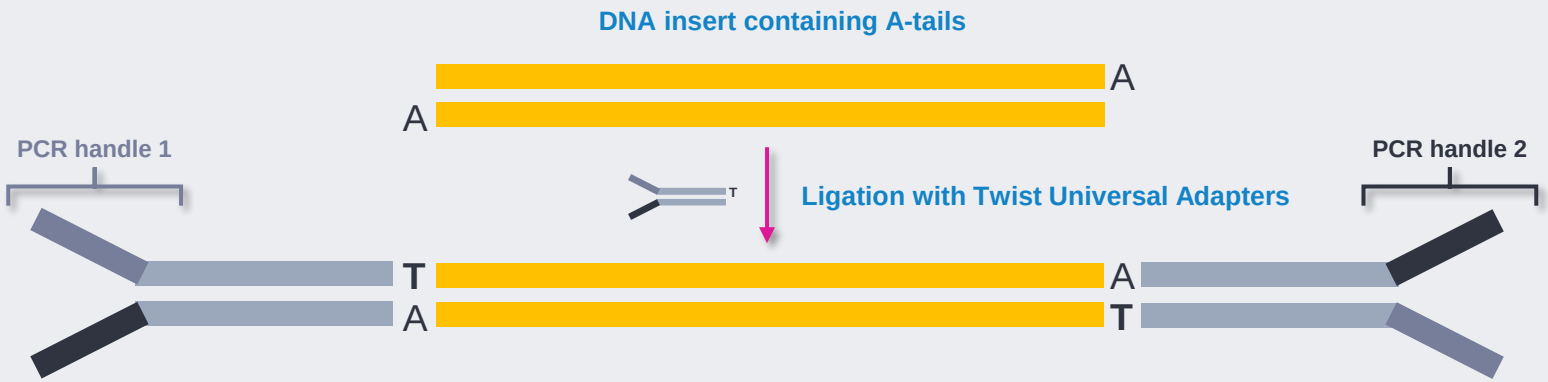
7 Nuclease treatment

8 AMPure PB bead size selection

Twist Universal Adapters provide handles for subsequent PCR amplification of DNA inserts



Twist Universal Adapter containing T-tail



Ligation of amplification adapter and cleanup

Procedure notes

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

3. Ligation of universal PCR amplification adapter and cleanup

✔	Step	Instructions for amplification adapter ligation and reaction cleanup
	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 mastermix

✔	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 mastermix calculation

	3.2	Pipette-mix the Ligation mastermix.
	3.3	Quick-spin the Ligation mastermix in a microcentrifuge to collect liquid.
	3.4	Add 18 µL of the Ligation mastermix containing the Twist universal adapter to each sample from the previous step. The total volume per sample should be 78 µL.

	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

	3.8	Add 78 µL of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	3.9	Pipette-mix the beads until evenly distributed.

- Prepare ligation rxn master mix containing **Universal Adapters** available from Twist Bioscience by adding required components in the order and volume listed to a new microcentrifuge tube

- Add **18 µL** of master mix to each sample
- Total universal adapter ligation rxn volume **may be up to 89 µL** (if using up to 60 µL of post-sheared DNA for Repair & A-tailing rxn in Step 2)

- Run **Adapter ligation** thermal cycler program
- Set lid temperature to **≥30°C** if programmable

- Perform **1X SMRTbell bead cleanup**
- **If needed**, adjust required vol. of SMRTbell cleanup beads based on total universal adapter ligation rxn vol.
- Elute cleaned DNA into **24 µL** of EB buffer

PacBio

Library amplification by PCR and cleanup

PCR-amplify genomic DNA fragments ligated with amplification adapters on both ends

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

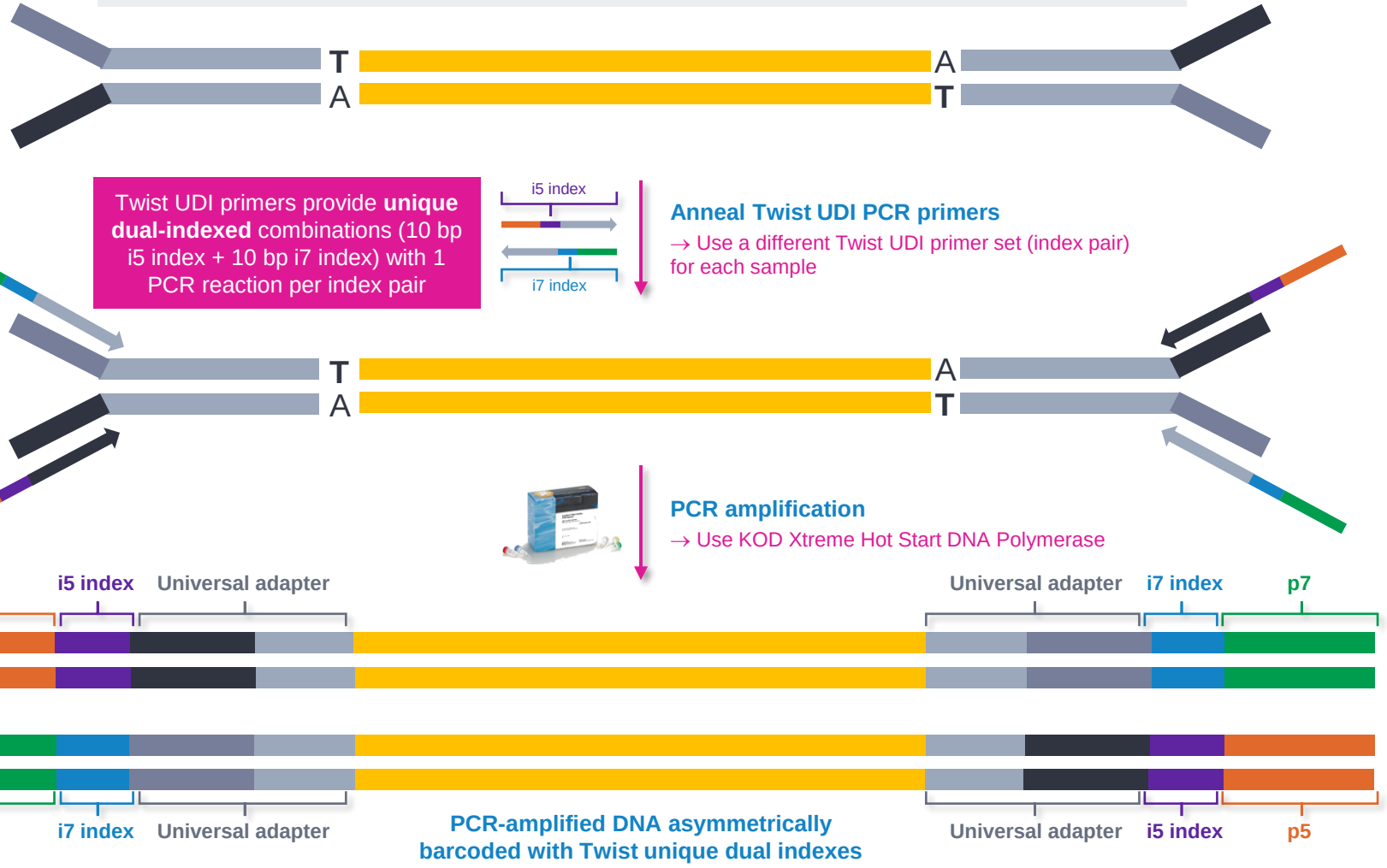
6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

Note: In this procedure, **all** Ampli-Fi samples are indexed (and asymmetrically barcoded) during PCR amplification step using Twist UDI primers that contain 10-bp i5 + 10-bp i7 indexes



Library amplification by PCR and cleanup

Procedural notes

QC

DNA Shearing & amplification

Library construction

- DNA sample extraction & QC
- 1 Shear DNA
- 2 Repair & A-tailing of sheared DNA
- 3 Ligation of amplification adapter and cleanup
- Safe stop
- 4 Library amplification by PCR and cleanup
- Safe stop
- 5 Repair & A-tailing of amplified DNA
- 6 SMRTbell adapter ligation and cleanup
- Safe stop
- 7 Nuclease treatment
- 8

- Perform **1X SMRTbell bead cleanup**
- Add **100 µL** SMRTbell cleanup beads to each (**100 µL**) PCR reaction
- Elute cleaned DNA into **50 µL** of EB

- Add **4 µL of** Twist UDI primer to each sample (**24 µL**) to bring sample + primer vol. to **28 µL**

4. Library amplification by PCR and cleanup

✔	Step	Instructions library amplification

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.

Amplification mastermix

✔	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**

4.3 Quick-spin the Amplification mastermix in a microcentrifuge to collect liquid.

4.4 On ice, add **72** µL of the Amplification mastermix to **28 µl of sample + Twist UDI primer solution** for a total volume of 100 µL.

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.

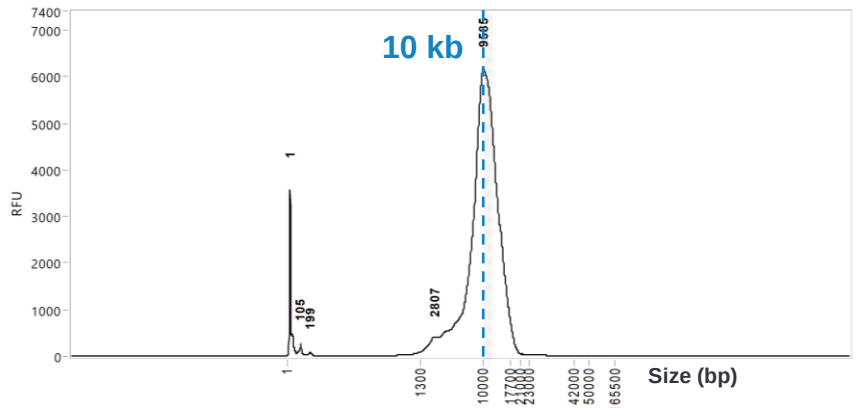
Step	Time	Temperature	Cycles
1	2 min	94°C	1 cycle
2	10 sec	98°C	8-14 cycles
3	30 sec	58.8°C	
4	10 min	68°C	
5	7 min	68°C	1 cycle
6	Hold	4°C	

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

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

- Prepare PCR rxn master mix by adding required components in the order and volume listed to a new tube
- Add **72 µL** of PCR master mix to each sample + primer solution (**28 µL**) for a total rxn volume of **100 µL**

- Run **PCR** thermal cycler program using recommended number of amplification cycles based on DNA input amount
- Set lid temperature to **105°C** if programmable
- Do not add samples to thermal cycler until lid has pre-heated



Example sheared human DNA sample amplified by PCR. Size distribution of amplified products is ~10 kb and appropriate to proceed to SMRTbell library construction.

IMPORTANT!

- You must have the required mass of purified amplified DNA per reaction to proceed with SMRTbell library prep
  - ≥150 ng or ≥600 ng of amplified DNA mass[1] is required to yield enough polymerase-bound Ampli-Fi library (10 kb) for sequencing using Revio SPRQ chemistry or Revio non-SPRQ/Vega chemistry (120 pM on-plate loading concentration).

[1] See Appendix section of procedure for guidance on running additional PCR cycles for samples with low PCR yield.

DNA repair & A-tailing of amplified DNA

Repair sites of DNA damage and prepare amplified DNA for ligation to SMRTbell adapter

QC
- DNA sample extraction & QC

DNA Shearing & amplification
1. Shear DNA
2. Repair & A-tailing of sheared DNA
3. Ligation of amplification adapter and cleanup
   - Safe stop
4. Library amplification by PCR and cleanup
   - Safe stop

Library construction
5. Repair & A-tailing of amplified DNA
6. SMRTbell adapter ligation and cleanup
   - Safe stop
7. Nuclease treatment
8. AMPure PB bead size selection

5. Repair and A-tailing of amplified DNA

✔	Step	Instructions for DNA damage and end repair of amplified DNA

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 mastermix

✔	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 mastermix calculations

5.2 Pipette-mix the Repair mastermix.

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

5.4 Add 11 µL of the Repair mastermix to **49 µl of sample** from step 4.21 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.

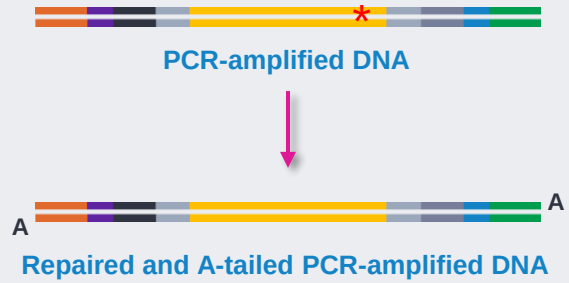
Step	Time	Temperature
1	30 min	37°C
2	5 min	65°C
3	Hold	4°C

5.8 Proceed to the next step of the protocol.

- Prepare a rxn master mix by adding required components in the order and volume listed to a new tube

Optional: If multiplexing, indexed amplified samples can be pooled prior to performing this repair and A-tailing step (Step 5) if desired. Alternatively, samples can be pooled at the end of the protocol after Step 9 (Annealing, binding, and cleanup – ABC) prior to sequencing.

* = DNA damage



- Add **11 µL** of master mix to **49 µL** of amplified DNA sample from Step 4 for a total rxn volume of **60 µL**

- Run **Repair and A-tailing** thermal cycler program
- Set lid temperature to **≥75°C** if programmable

PacBio

Adapter ligation & cleanup

Ligate SMRTbell adapter to the ends of each DNA fragment

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

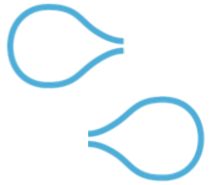
8 AMPure PB bead size selection

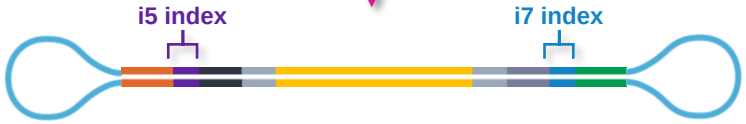
Repaired and A-tailed PCR-amplified DNA

Ligation with non-indexed SMRTbell adapters (RECOMMENDED)

→ **RECOMMENDED: All** Ampli-Fi samples are **already indexed** (and asymmetrically barcoded) during PCR amplification step using Twist UDI primers and therefore **do not require** the use of a SMRTbell adapter index for sample multiplexing applications

Use **non-indexed SMRTbell adapters** included in SMRTbell prep kit 3.0





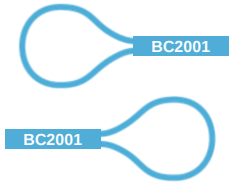
SMRTbell library containing non-indexed SMRTbell adapters and Twist unique dual indexes

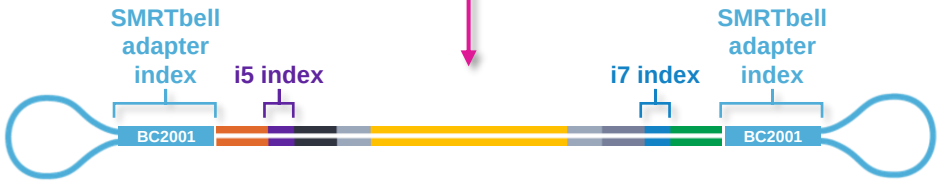
Or

Ligation with indexed SMRTbell adapters (OPTIONAL)

→ **OPTIONAL: If** desired, ligation with indexed SMRTbell adapters provides an **additional method of symmetrically barcoding samples**[1] when preparing multiplexed Ampli-Fi libraries

Use **indexed SMRTbell adapters** included in SMRTbell indexed adapter plate 96A/B/C/D





SMRTbell library containing indexed SMRTbell adapters and Twist unique dual indexes

[1] If barcoding Ampli-Fi samples using SMRTbell adapter index plate 96A/B/C/D, then two rounds of barcode demultiplexing are required: First perform barcode demultiplexing **on-instrument** to demultiplex adapter-barcoded samples; and then proceed to run Demultiplex Barcodes **in SMRT Link** to trim Twist universal adapters and demultiplex Twist UDIs (refer to Ampli-Fi data analysis workflow recommendations described in Section 4 of this presentation).

Adapter ligation & cleanup

Procedural notes

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

6. SMRTbell adapter ligation & cleanup

✔	Step	Instructions for SMRTbell adapter ligation and reaction cleanup
		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 mastermix (next step). One index per SMRTbell adapter index plate well per sample.

6.2 **Ligation mastermix**

✔	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.

6.5
- No barcoding: add 20 µL of the Ligation mastermix containing the SMRTbell adapter to each sample. The total volume per sample should be 80 µL.
- Barcoding: add 16 µL of the Ligation mastermix containing to each sample.

The total volume per sample should be 80 µL.

6.8 Run the Adapter ligation thermocycler program.

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

6.9 Add 80 µL of resuspended, room-temperature SMRTbell cleanup beads to each sample.

6.10 Pipette-mix the beads until evenly distributed.

- **Optional if using indexed SMRTbell adapters to barcode samples:** Add **4 µL** of indexed adapter (from SMRTbell adapter index plate 96A/B/C/D) to each sample **(60 µL)** from Step 5 to bring the sample + indexed adapter volume to **64 µL**
- **Skip this step if *not* using an adapter index to barcode your sample**

- Prepare a rxn master mix by adding required components in the order and volume listed to a new microcentrifuge tube
- ***IMPORTANT!:* Exclude the SMRTbell adapter from the master mix *if* using an indexed adapter to barcode your sample**

- **If using non-indexed SMRTbell adapters:** Add 20 **µL** of master mix to each sample (**60 µL**) for total rxn vol. = **80 µL**
- **If using indexed SMRTbell adapters:** Add **16 µL** of master mix to each sample + indexed adapter (**64 µL**) for total rxn vol. = **80 µL**

- Run **Adapter ligation** thermal cycler program
- Set the lid temperature to **≥30°C** if programmable

- Perform **1X SMRTbell bead cleanup**
- Add **80 µL** of SMRTbell cleanup beads to each (**80 µL**) SMRTbell adapter ligation reaction
- Elute cleaned DNA into **40 µL** of EB buffer

PacBio

Nuclease treatment

Remove unligated DNA fragments and leftover SMRTbell adapters from the sample

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

7. Nuclease treatment

✔	Step	Instructions for nuclease treatment
	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.

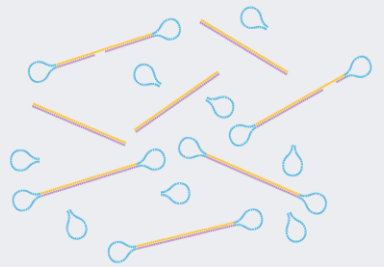
Nuclease mastermix

✔	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 mastermix.
	7.3	Quick-spin the Nuclease mastermix in a microcentrifuge to collect liquid.
	7.4	Add 10 µL of Nuclease mastermix 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.

Ste	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 cleanup the nuclease reaction using the AMPure PB or SMRTbell cleanup beads prior to safely storing the library or libraries.



Sample containing incomplete SMRTbell templates and unligated DNA fragments

Sample containing mostly intact SMRTbell templates

- Prepare a rxn master mix by adding required components in the order and volume listed to a new tube
- Add **10 µL** of master mix to **40 µL** of adapter-ligated DNA sample from Step 6 for a total rxn volume of **50 µL**
- Run **Nuclease treatment** thermal cycler program
- Set the lid temperature to **≥47°C** if programmable
- **Note:** It is necessary to remove nucleases using either **AMPure PB size selection *or* SMRTbell cleanup beads (Step 8)** prior to safely storing the library or libraries.

PacBio

Diluted AMPure PB cleanup and size selection

AMPure PB bead size cleanup and selection step will clean the library and progressively deplete DNA fragments <5 kb

QC

DNA sample extraction & QC

DNA Shearing & amplification

1 Shear DNA

2 Repair & A-tailing of sheared DNA

3 Ligation of amplification adapter and cleanup

Safe stop

4 Library amplification by PCR and cleanup

Safe stop

Library construction

5 Repair & A-tailing of amplified DNA

6 SMRTbell adapter ligation and cleanup

Safe stop

7 Nuclease treatment

8 AMPure PB bead size selection

8. Diluted AMPure PB cleanup and size selection

✔	Step	Instructions for AMPure PB bead size selection
	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.
	8.15	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.

- ***IMPORTANT!:*** If performing gel-based size selection or if DNA library insert size is ~3 – 5 kb:
  → Skip diluted AMPure PB bead size selection and perform a cleanup using 1X SMRTbell cleanup beads instead[1]

- Prepare a **35% (v/v) dilution of AMPure PB beads** using elution buffer and add **3.1X (155 µL)** of diluted beads to sample (**50 µL**)[2]
  - 35% AMPure PB solution can be stored at **4°C for 30 days**
- **Note:** The AMPure PB dilution procedure may be scaled as appropriate for smaller-/larger-scale projects (each sample requires 155 µL of 35% AMPure PB beads)

- After eluting in **25 µL** of EB, perform **DNA concentration QC** on final Ampli-Fi library using Qubit assay
- Recommend to perform DNA sizing QC using Femto Pulse

- **Note:** Final Ampli-Fi library concentration must be **<20 ng/ µL** for 3-10 kb insert sizes and **<10 ng/ µL** for inserts <3 kb to proceed with annealing, binding & cleanup (ABC)
  → Using a concentration higher than recommended for ABC may negatively impact sequencing performance

- To prepare Ampli-Fi samples for sequencing using Revio SPRQ or Vega chemistry, follow ABC workflow instructions in Step 9 using the recommended loading concentration

PacBio

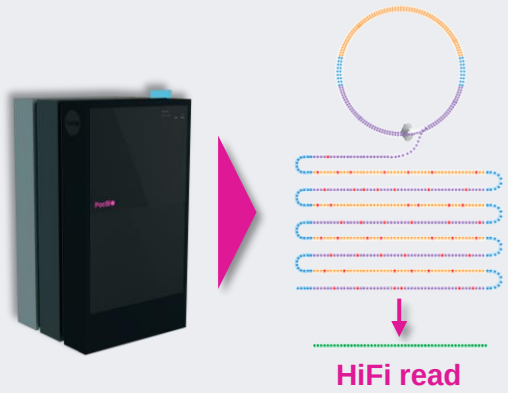
[1] If DNA library insert size is <3 kb, then replace all 1X bead cleanup steps in the Ampli-Fi library prep procedure with 1.3X bead cleanups.

[2] ***IMPORTANT!:*** 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.

Ampli-Fi library sequencing preparation workflow details

Sample Setup & Run Design recommendations for SPK 3.0 Ampli-Fi libraries – Revio system

Workflow	Key setup parameters	Revio system recommended settings
		Ampli-Fi samples
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Revio (non-SPRQ) polymerase kit / Revio SPRQ polymerase kit
	Concentration on plate (OPLC)	120 – 160 pM
Run design	Library type	Standard
	Movie acquisition time	24 hrs (~5 – 20 kb)
	Use adaptive loading	YES
	Data options[1]	Include base kinetics = NO[1] Consensus Mode = MOLECULE



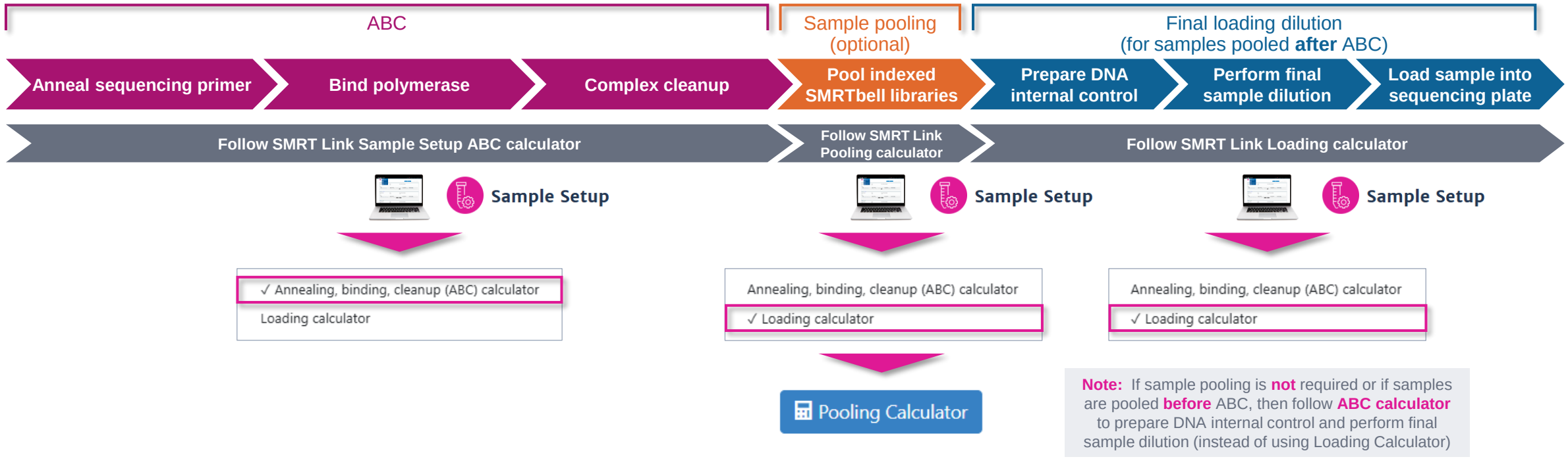
Recommended target *P1* loading range for Revio system is **~50 – 70%**

[1] **IMPORTANT:** If analysis of 4mC, 6mA or other custom modifications is desired to be performed in SMRT Link, then need to specify **Include Base Kinetics = YES**.

Sample setup workflow overview for Revio (non-SPRQ) polymerase libraries

For binding libraries with Revio (non-SPRQ) polymerase kit, follow SMRT Link Sample Setup ABC calculator instructions for annealing/binding/complex cleanup steps

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 Ampli-Fi	**Revio (non-SPRQ) polymerase kit** (102-817-600)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link Sample Setup ABC calculator
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator (if pooling samples **after** ABC)[1]



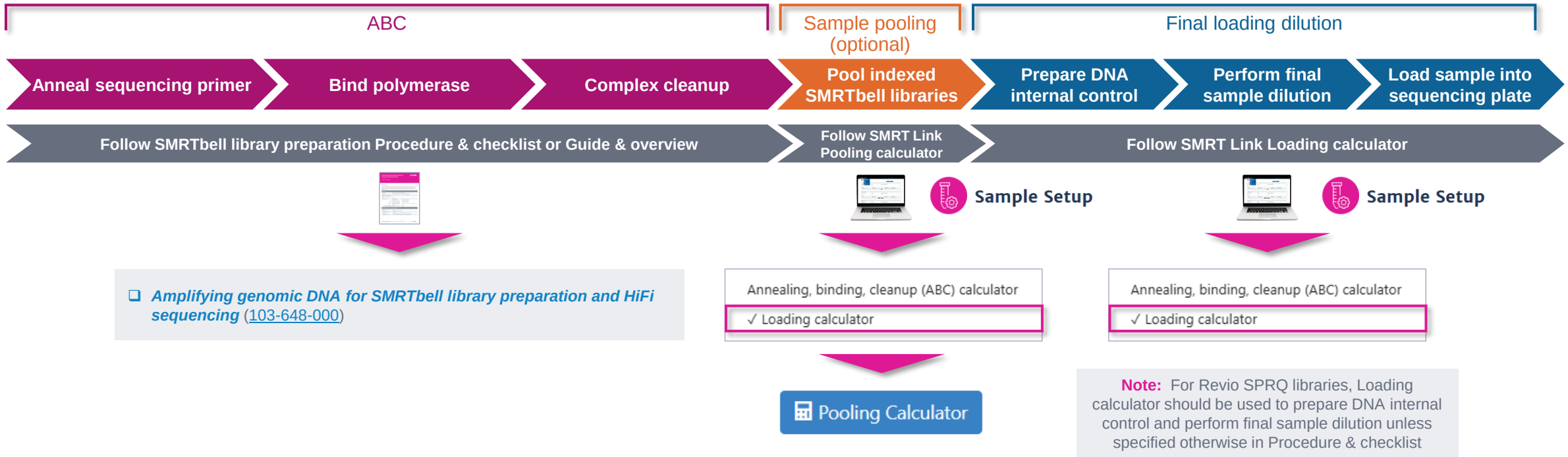
Note: If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator)

[1] If sample pooling is **not** required or if samples are pooled **before** ABC, then follow **ABC calculator** to prepare DNA internal control and perform final sample dilution (instead of using Loading Calculator).

Sample setup workflow overview for Revio SPRQ polymerase libraries

For binding libraries with Revio SPRQ polymerase kit, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

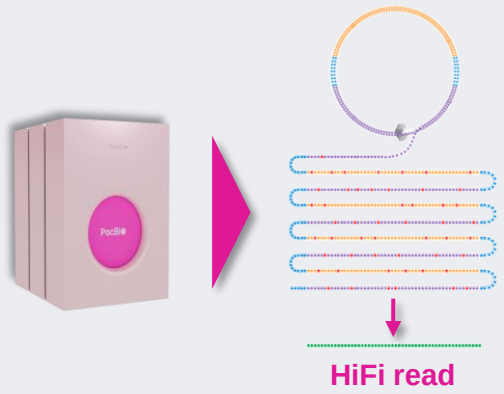
Library type[1,2]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 Ampli-Fi	**Revio SPRQ polymerase kit** (103-520-100)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator



Note: For Revio SPRQ libraries, Loading calculator should be used to prepare DNA internal control and perform final sample dilution unless specified otherwise in Procedure & checklist

Sample Setup & Run Design recommendations for SPK 3.0 Ampli-Fi libraries – Vega system

Workflow	Key setup parameters	Vega system recommended settings
		Ampli-Fi samples
Sample setup	Library type	Standard
	Primer	Standard sequencing primer
	Polymerase kit	Vega polymerase kit
	Concentration on plate (OPLC)	100 – 140 pM
Run design	Library type	Standard
	Movie acquisition time	24 hrs (~7 – 10 kb)
	Use adaptive loading	YES
	Data options[1]	Include base kinetics = NO[1] Consensus Mode = MOLECULE



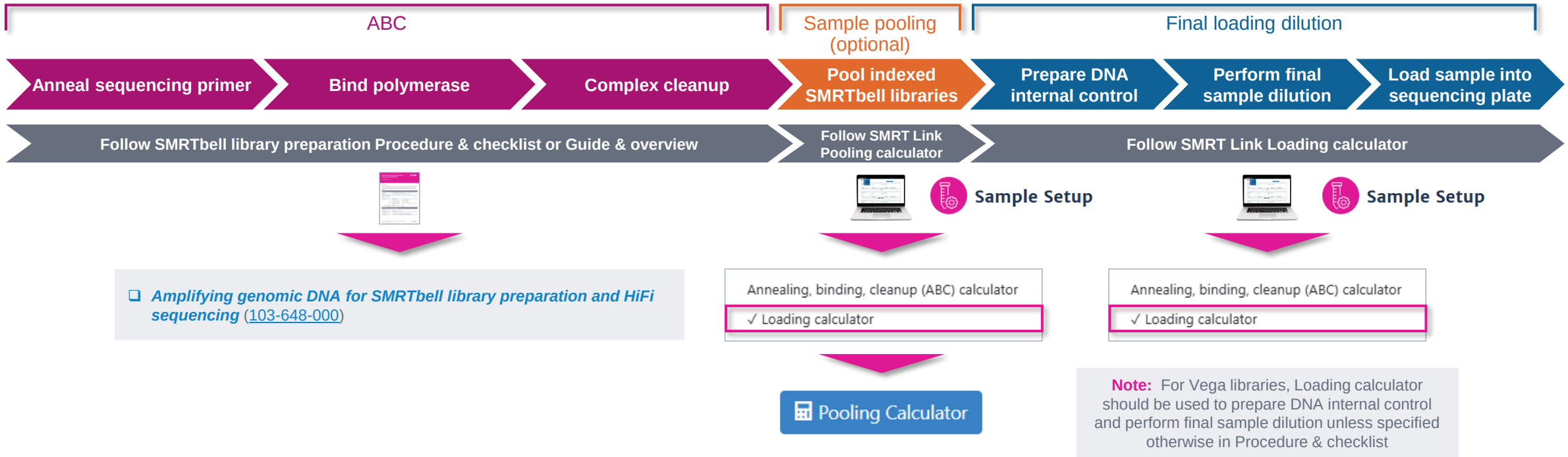
Recommended target loading level range for Vega system is **~50 – 70%**

[1] **IMPORTANT:** If analysis of 4mC, 6mA or other custom modifications is desired to be performed in SMRT Link, then need to specify **Include Base Kinetics = YES**.

Sample setup workflow overview for Vega polymerase libraries

For binding libraries with Vega polymerase kit, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1,2]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 Ampli-Fi	Vega polymerase kit (103-520-100)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling (optional)	❑ Follow SMRT Link Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link Sample Setup Loading calculator



General best practices recommendations for preparing SPK 3.0 Ampli-Fi libraries for sequencing on Revio and Vega systems

Polymerase kit thawing procedure[1]

Revio polymerase kit / Revio SPRQ polymerase kit / Vega polymerase kit

Revio SPRQ polymerase kit
(103-520-100)

Vega polymerase kit
(103-517-600)

Thaw these reagents at room temperature		Keep these reagents on a cold block or on ice		Bring these reagents to room temperature 30 minutes prior to use	
	Annealing buffer		Sequencing polymerase		Loading buffer
	Standard sequencing primer		Sequencing control		SMRTbell cleanup beads
	Polymerase buffer				
	Loading buffer				
	Dilution buffer				

- Once thawed, reaction buffers and sequencing primer may be stored on a cold block, at 4°C, or on-ice prior to making master mix or placing on the liquid handler work deck
- Loading buffer should be left at room-temperature
- **Note:** Loading buffer is light sensitive and should be protected from light when not in use

[1] Refer to *Procedure & checklist* protocol for detailed instructions for handling all required sequencing preparation reagents.

Ampli-Fi data analysis recommendations for supported applications & use cases

Ampli-Fi data analysis workflow recommendations

Comparison of SMRT Link data analysis workflows for Ampli-Fi library HiFi data sets *versus* Ultra-low DNA input library HiFi data sets

Ampli-Fi libraries

Generate Ampli-Fi HiFi data on PacBio sequencer

Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)

In SMRT Link
`Demultiplex Barcodes`

Remove Twist universal adapters and **demultiplex Twist UDI primer sequences** used for PCR amplification step

In SMRT Link
`Mark PCR Duplicates`

Remove PCR duplicate reads

Trimmed and de-duplicated Ampli-Fi HiFi data

Perform secondary analysis (e.g., assembly or variant calling)

Use **SMRT Link** or standard **3rd-party tools** to perform assembly or variant calling

Ultra-low DNA input libraries

Generate Ultra-low DNA input HiFi data on PacBio sequencer

Procedure & checklist – Preparing HiFi Libraries from Ultra-Low DNA Input (101-995-900)

In SMRT Link
`Trim Ultra-Low Adapters`

Remove ultra-low DNA input adapter sequences used for PCR amplification step

In SMRT Link
`Mark PCR Duplicates`

Remove PCR duplicate reads

Trimmed and de-duplicated Ultra-low DNA input HiFi data

Perform secondary analysis (e.g., assembly or variant calling)

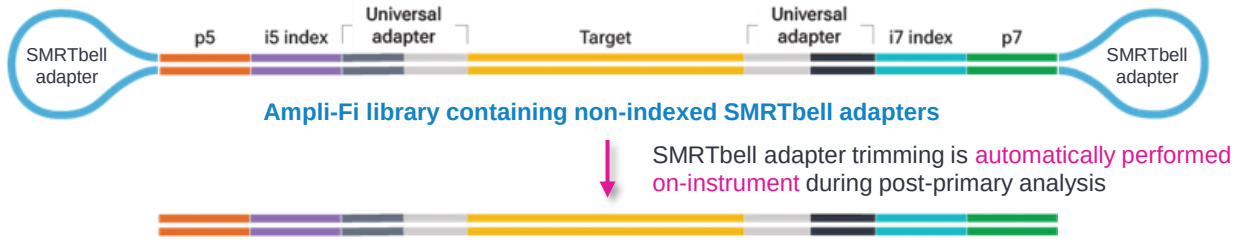
Use **SMRT Link** or standard **3rd-party tools** to perform assembly or variant calling

Ampli-Fi data preparation workflow

Use SMRT Link data utilities to prepare Ampli-Fi HiFi data for downstream secondary analysis applications by trimming PCR amplification adapters and removing PCR duplicate reads

Data preparation workflow for Ampli-Fi libraries containing non-indexed SMRTbell adapters

Generate Ampli-Fi HiFi data on PacBio sequencer



Ampli-Fi library containing non-indexed SMRTbell adapters

SMRTbell adapter trimming is automatically performed on-instrument during post-primary analysis

Ampli-Fi HiFi read containing Twist universal adapter & Twist unique dual indexed (UDI) primer sequences

Trim Twist universal adapters and demultiplex Twist UDIs

Run '**Demultiplex Barcodes** ' data utility job in SMRT Link or use `lima` command line tool to remove Twist universal adapters and demultiplex Twist UDI primer sequences

Ampli-Fi HiFi read with Twist universal adapter & Twist UDI primer sequences removed

Remove PCR duplicate reads

Run '**Mark PCR Duplicates**' data utility job in SMRT Link or use `pbmarkdups` command line tool[1] (pbbioconda) to remove PCR duplicate reads

Trimmed and de-duplicated Ampli-Fi HiFi data

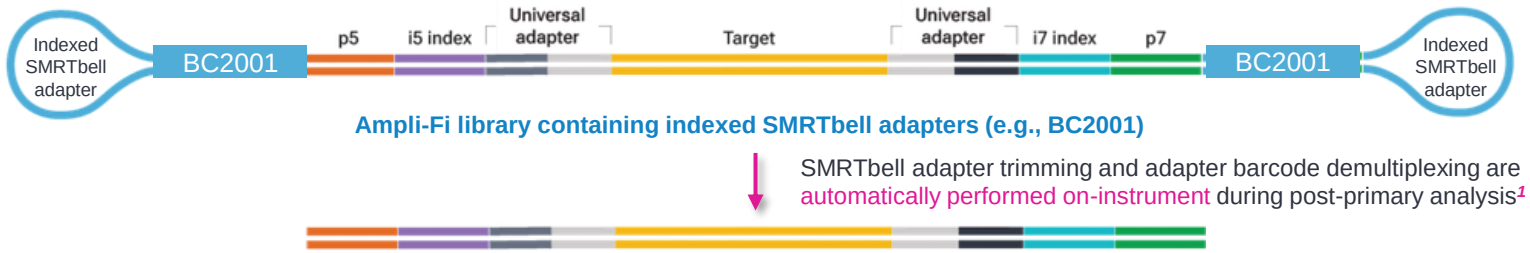
[1] **Note:** If running SMRT Link v25.1 or earlier, then users may need to run Mark PCR Duplicates using the command line tool in order to be able to provide additional memory resources for the analysis.

Ampli-Fi data preparation workflow (cont.)

Use SMRT Link data utilities to prepare Ampli-Fi HiFi data for downstream secondary analysis applications by trimming PCR amplification adapters and removing PCR duplicate reads

Data preparation workflow for Ampli-Fi libraries containing indexed SMRTbell adapters

Generate Ampli-Fi HiFi data on PacBio sequencer



Ampli-Fi library containing indexed SMRTbell adapters (e.g., BC2001)

SMRTbell adapter trimming and adapter barcode demultiplexing are automatically performed on-instrument during post-primary analysis[1]

Ampli-Fi HiFi read containing Twist universal adapter & Twist unique dual indexed (UDI) primer sequences

Trim Twist universal adapters and demultiplex Twist UDIs

Run '**Demultiplex Barcodes** ' data utility job in SMRT Link or use `lima` command line tool to remove Twist universal adapters and demultiplex Twist UDI primer sequences

Ampli-Fi HiFi read with Twist universal adapter & Twist UDI primer sequences removed

Remove PCR duplicate reads

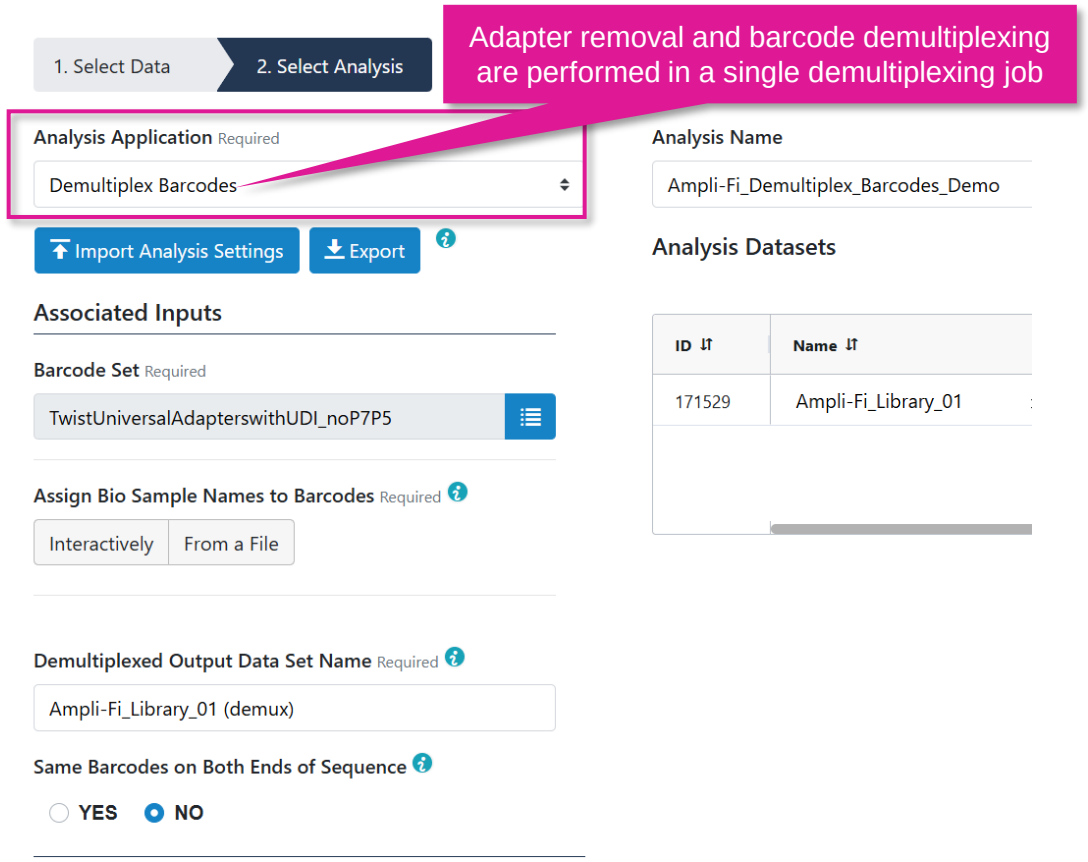
Run '**Mark PCR Duplicates**' data utility job in SMRT Link or use `pbmarkdups` command line tool[2] (pbbioconda) to remove PCR duplicate reads

Trimmed and de-duplicated Ampli-Fi HiFi data

[1] **Note:** To enable automatic adapter barcode demultiplexing on-instrument, specify '**Sample is indexed = YES**' in the sequencing run design.
[2] **Note:** If running SMRT Link v25.1 or earlier, then users may need to run Mark PCR Duplicates using the command line tool in order to be able to provide additional memory resources for the analysis.

SMRT Link Demultiplex Barcodes data utility for Ampli-Fi data preparation

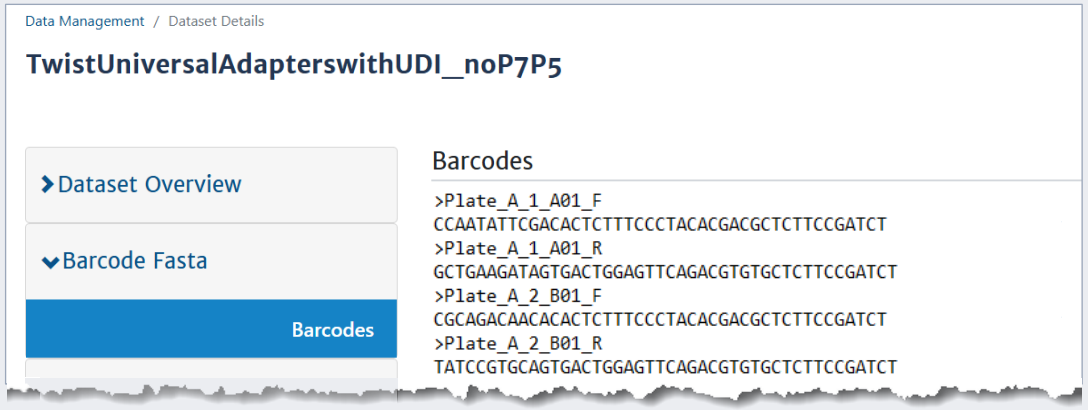
Use SMRT Link Demultiplex Barcodes data utility to trim Twist universal adapters and Twist UDI primer sequences from an Ampli-Fi HiFi data set



Schematic of Ampli-Fi library structure containing Twist universal adapter and Twist UDI primer sequences

`Amplifi_TwistUDIadapters_noP7P5` [Link] barcode set FASTA includes the Twist universal adapter and UDI sequences (and does not include any P5/P7 sequences)[1]

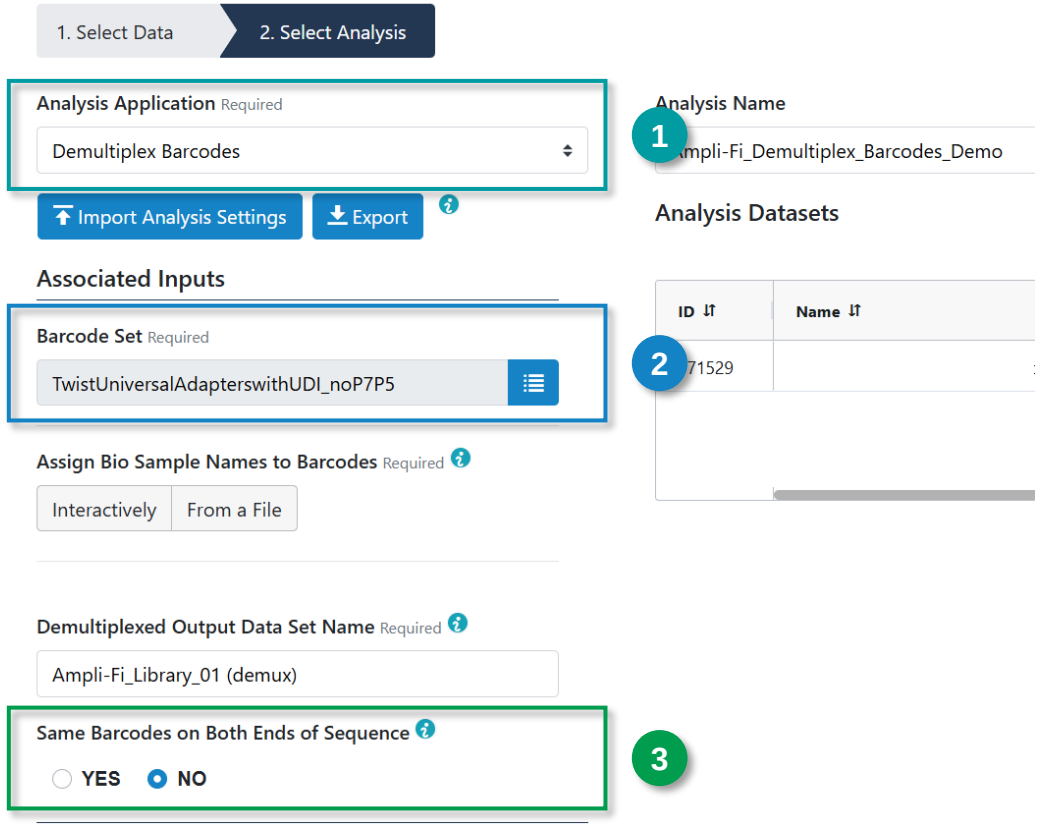
```
UDI<------- Universal adapter ----->
[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT

UDI<------- Universal adapter ----->
[i7]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
```



[1] **Note:** The `Twist Universal Adapters with UDI` barcode set preloaded in SMRT Link v25.1 and v25.2 contains sequences corresponding to the P5/P7 adapters, UDIs, **and** universal adapters. Because the Ampli-Fi library procedure can result in truncated P5/P7 adapter sequences, for optimal barcode demultiplexing performance we recommend using the `Amplifi_TwistUDIadapters_noP7P5` barcode set FASTA [Link], which contains **only** the UDI and universal adapter sequences (and omits the P5/P7 adapter sequences).

SMRT Link Demultiplex Barcodes data utility for Ampli-Fi data preparation (cont.)

Follow procedure below to trim Twist universal adapters and Twist UDI primer sequences from an Ampli-Fi HiFi data set using SMRT Link Demultiplex Barcodes data utility



SMRT Link → SMRT Analysis → Create New Job

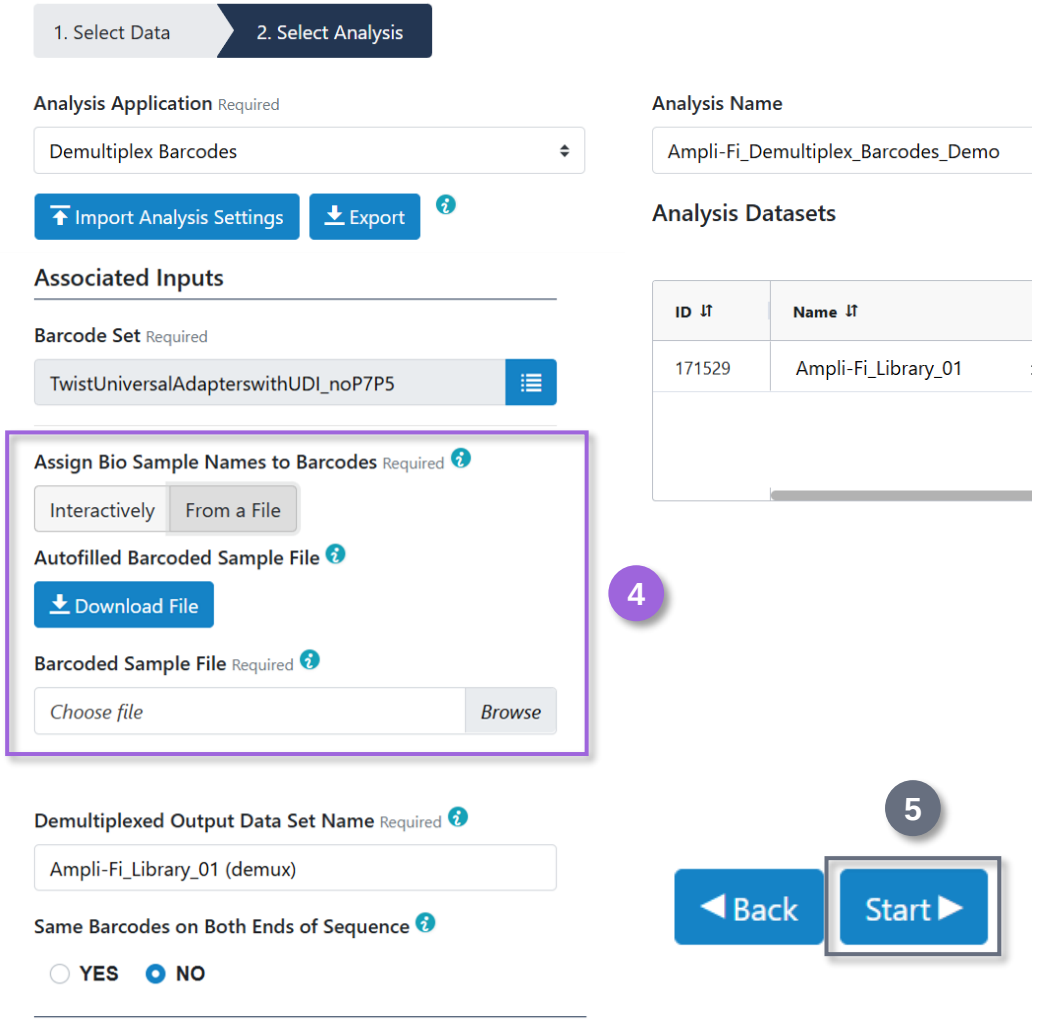
1. **After selecting your data set, specify analysis application**
   - Select **Demultiplex Barcodes**
2. **Specify barcode set**
   - Click on **Barcode Set** selection button and select recommended barcode set FASTA: **`Amplifi_TwistUDIadapters_noP7P5`**
   - If using SMRT Link v25.2 or earlier, download recommended barcode set FASTA from PacBio Multiplexing Resources website:
     - ❑ **`Amplifi_TwistUDIadapters_noP7P5`** [Link]

     After downloading recommended barcode set FASTA, import the file into SMRT Link using Data Management module
3. **Specify if using same barcodes on both ends of sequence**
   - Specify **NO**

Continued on next page…

SMRT Link Demultiplex Barcodes data utility for Ampli-Fi data preparation (cont.)

Follow procedure below to trim Twist universal adapters and Twist UDI primer sequences from an Ampli-Fi HiFi data set using SMRT Link Demultiplex Barcodes data utility



4. Assign bio sample names to barcodes

- First download the recommended barcoded sample CSV template file from PacBio Multiplexing Resources website:
  - ❑ `Ampli-Fi_Barcoded_Sample_Name_File.csv` [Link]
- After downloading the CSV template file, fill out the biological sample names[1] for each barcode used in the "Bio Sample Name" column, and delete rows of unused barcodes. Then save the edited CSV file.

Barcode	Bio Sample Name
Plate_A_1_A01_F--Plate_A_1_A01_R	Ampli-Fi_library_01
Plate_A_2_B01_F--Plate_A_2_B01_R	Ampli-Fi_library_02
Plate_A_3_C01_F--Plate_A_3_C01_R	Ampli-Fi_library_03
Plate_A_4_D01_F--Plate_A_4_D01_R	Ampli-Fi_library_04

- Click on the 'From a File' button and Click "Browse", find the edited file, then click "**Open**" to upload it. '**Upload was successful**' appears if file is formatted correctly.

Upload was successful

5. Click on **Start** button

- Demultiplex Barcodes utility job will immediately begin

[1] **Note:** Bio Sample names must be 40 characters or fewer limited to: Only use alphanumeric; dot; underscore; hyphen. Do not include spaces – Sample names must be unique and will be truncated after any spaces. Delete any barcodes not used in the CSV file.

Example Twist UDI barcode demultiplexing performance for Ampli-Fi libraries[1]

Demultiplexing rates are typically lower for Ampli-Fi libraries containing Twist UDI barcodes compared to non-amplified (symmetrically) barcoded gDNA libraries containing indexed SMRTbell adapters

Revio system with SPRQ chemistry

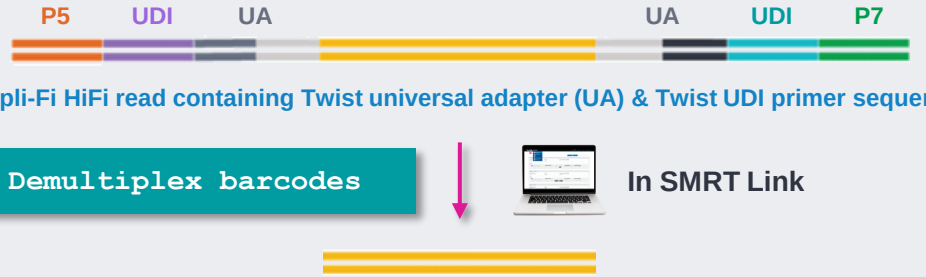
Sample	Twist UDI	Barcoded HiFi Reads	Barcoded HiFi Yield	Barcode Quality
MR_1ng_hg2_65uL_sp59	PlateA_25	90.9%	69.96 Gb	98.4
MR_5ng_hg2_65uL_sp59	PlateA_27	93.0%	73.74 Gb	98.5
MR_20ng_hg2_65uL_sp59	PlateA_29	94.3%	63.20 Gb	98.8
MR_50ng_hg2_65uL_sp59	PlateA_31	92.6%	77.69 Gb	98.1

Sample	Twist UDI	Barcoded HiFi Reads	Barcoded HiFi Yield	Barcode Quality
gTube_1ng_PCR14_S13	PlateA_77	90.5%	54.66 Gb	98.5
gTube_5ng_PCR12_S14	PlateA_78	93.4%	56.77 Gb	98.6
gTube_20ng_PCR10_S15	PlateA_79	94.9%	51.57 Gb	98.7
gTube_50ng_PCR08_S16	PlateA_80	92.6%	58.75 Gb	98.9

Vega system

Sample	Twist UDI	Barcoded HiFi Reads	Barcoded HiFi Yield	Barcode Quality
MR_1ng_hg2_65uL_sp59	PlateA_26	90.1%	33.70 Gb	98.0
MR_5ng_hg2_65uL_sp59	PlateA_28	92.2%	36.56 Gb	98.4
MR_20ng_hg2_65uL_sp59	PlateA_30	93.8%	42.33 Gb	98.5
MR_50ng_hg2_65uL_sp59	PlateA_32	93.2%	40.41 Gb	98.9

Sample	Twist UDI	Barcoded HiFi Reads	Barcoded HiFi Yield	Barcode Quality
gTube_1ng_PCR14_S13	PlateA_77	90.7%	57.00 Gb	98.4
gTube_5ng_PCR12_S14	PlateA_78	92.7%	56.39 Gb	98.5
gTube_20ng_PCR10_S15	PlateA_79	94.7%	60.15 Gb	98.6
gTube_50ng_PCR08_S16	PlateA_80	92.1%	58.83 Gb	98.8

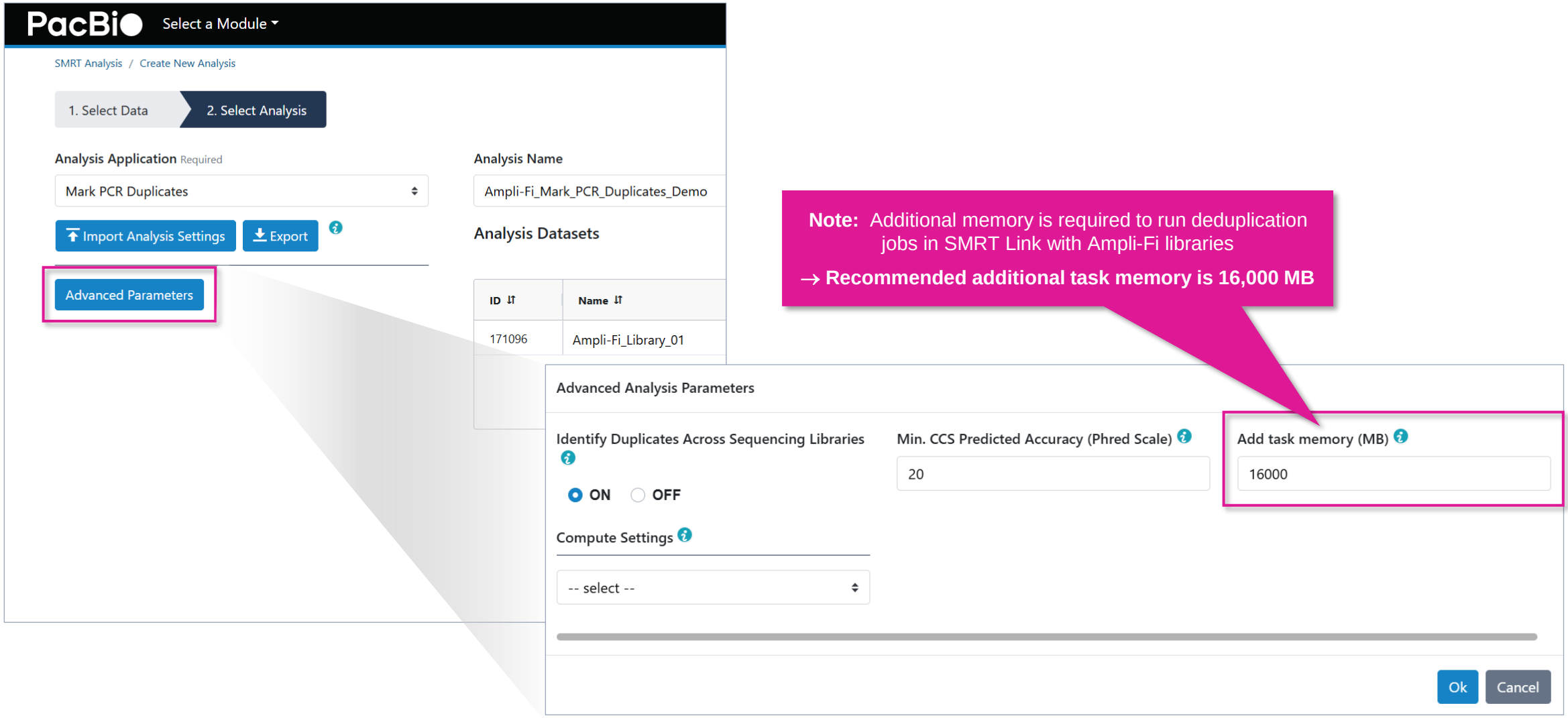


Note: Ampli-Fi DNA libraries amplified and asymmetrically barcoded with Twist UDI primers typically show lower barcode demultiplexing yields (~90 – 94%) compared to non-amplified symmetrically barcoded gDNA libraries containing indexed SMRTbell adapters due to replication errors and formation of truncated products during PCR amplification of long DNA templates

[1] Example barcode demultiplexing results shown are for Ampli-Fi libraries generated from high-quality human HG002 DNA samples and demultiplexed in SMRT Link using the Amplifi_TwistUDIadapters_noP7P5 barcode set file [Link]

SMRT Link Mark PCR Duplicates data utility for Ampli-Fi data preparation

Use SMRT Link Mark PCR Duplicates to remove duplicate reads from an Ampli-Fi HiFi data set



[1] **Note: Add task memory (MB)** option is available in SMRT Link v25.2 GUI but is not available in SMRT Link v25.1 GUI. If running SMRT Link v25.1 or earlier, then users may need to run Mark PCR Duplicates using the command line tool in order to be able to provide additional memory resources for the analysis.

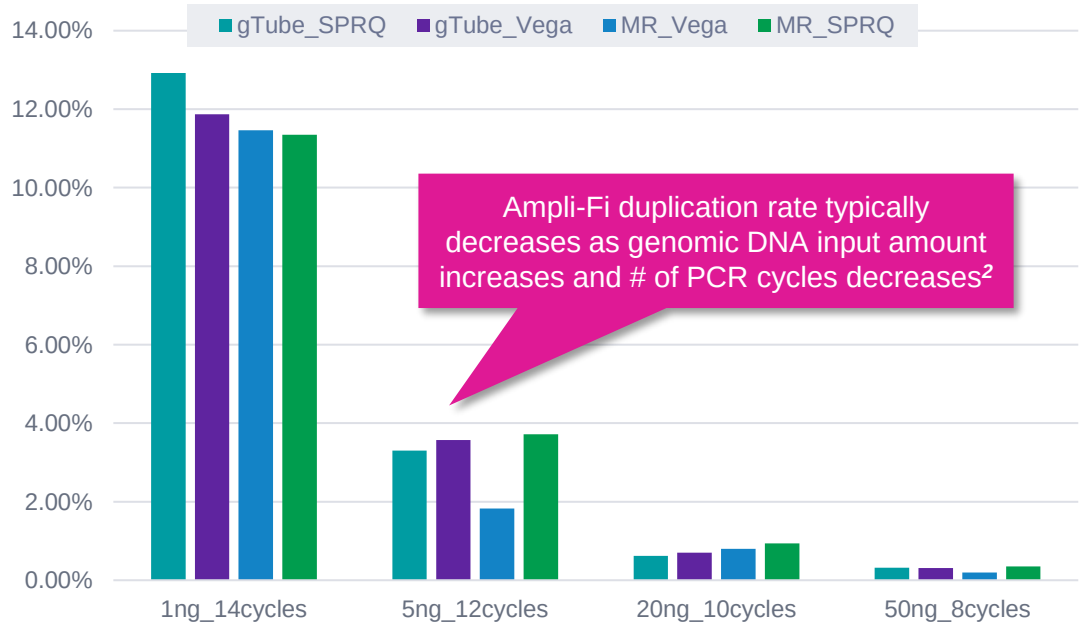
Example PCR duplication rates for Ampli-Fi libraries[1]

Ampli-Fi duplication rate typically decreases as genomic DNA input amount increases and # of PCR cycles decreases

gDNA input & # PCR cycles	g-TUBE Revio SPRQ	g-TUBE Vega	Megaruptor 3 Vega	Megaruptor 3 Revio SPRQ
1ng_14cycles	12.92%	11.87%	11.46%	11.35%
5ng_12cycles	3.30%	3.57%	1.83%	3.72%
20ng_10cycles	0.62%	0.70%	0.80%	0.94%
50ng_8cycles	0.32%	0.31%	0.20%	0.35%

Depending on gDNA input amount and # of PCR cycles used, PCR duplication rate ranges from ~0.3 – 12%

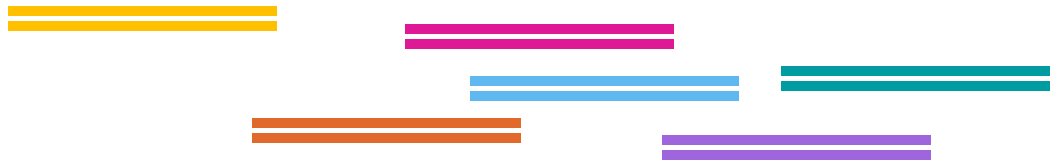
Ampli-Fi HiFi data set containing PCR-duplicate reads



Ampli-Fi duplication rate typically decreases as genomic DNA input amount increases and # of PCR cycles decreases[2]

Mark PCR duplicates

In SMRT Link



De-duplicated Ampli-Fi HiFi data set

PacBio

[1] Example PCR duplication rates shown are for Ampli-Fi libraries generated from high-quality human HG002 DNA samples.
[2] To achieve lower duplication rates, we generally recommend using higher gDNA inputs whenever possible. If using Revio SPRQ chemistry, # of PCR amplification cycles may be reduced further.

Ampli-Fi data analysis recommendations for *de novo* assembly

Using Ampli-Fi HiFi data for *de novo* assembly analysis of genomes

- **≥15-fold HiFi read coverage per haplotype** is recommended for most *de novo* assembly projects

  → $Target\ HiFi\ Base\ Yield = [Haploid\ Genome\ Size\ (Gb)]\ x\ [Ploidy\ Level]\ x\ [Target\ HiFi\ Coverage\ per\ Haplotype]$

  E.g., for *de novo* assembly analysis of a 3 Gb diploid genome:

  Recommended minimum target HiFi base yield = 3 Gb x 2 x 15 = 90 Gb

- Can use third-party software (e.g., Hifiasm) for *de novo* assembly analysis using HiFi reads:[1]

Note: Ampli-Fi samples require higher coverage levels compared to standard non-amplified genomic libraries for assembly and variant calling applications

[1] Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations.

Ampli-Fi data analysis recommendations for variant detection

Using Ampli-Fi HiFi data for variant detection analysis of genomes

- For detection of structural variants, we recommend **≥10-fold HiFi read coverage per sample**

  → $Target\ HiFi\ Base\ Yield = [Sample\ Haploid\ Genome\ Size\ (Gb)]\ x\ [Target\ Coverage\ per\ Sample]$

  E.g., For structural variant detection analysis of a large genome (3 Gb):

  Recommended minimum target HiFi base yield = 3 Gb x 10 = 30 Gb

- For detection of all variant classes, we recommend **≥20-fold HiFi read coverage per sample**

  → $Target\ HiFi\ Base\ Yield = [Sample\ Haploid\ Genome\ Size\ (Gb)]\ x\ [Target\ Coverage\ per\ Sample]$

  E.g., For detection of all variant classes in a large genome (3 Gb):

  Recommended minimum target HiFi base yield = 3 Gb x 20 = 60 Gb

- Recommend using sawfish GitHub tool (available through command line interface) for structural variant calling applications.
- Also compatible with SMRT Link Variant Calling analysis application (powered by Google DeepVariant & PacBio pbsv) for detection of small variants (SNVs, InDels)[1]

Note: Ampli-Fi samples require higher coverage levels compared to standard non-amplified genomic libraries for assembly and variant calling applications

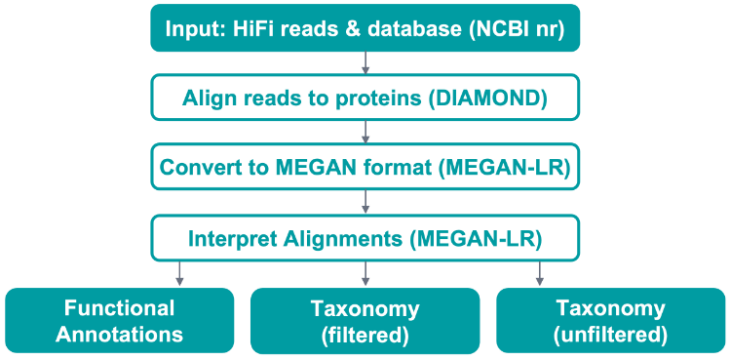
[1] Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations.

Ampli-Fi data analysis recommendations for shotgun metagenomics

Ampli-Fi HiFi data are compatible with 3rd-party metagenomics analysis tools for taxonomic & functional profiling

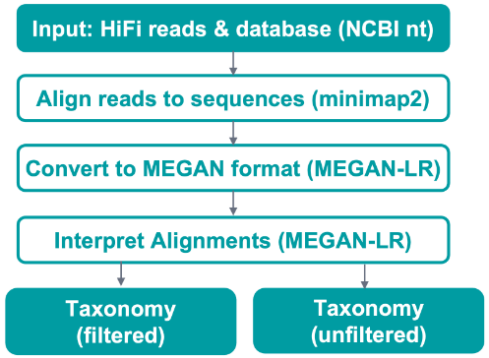
- Use SMRT Link to output HiFi data in standard file formats (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- Recommend using PacBio metagenomics tools available on GitHub for taxonomic classification and functional gene profiling using HiFi reads[1]

Taxonomic-Profiling-Diamond-Megan



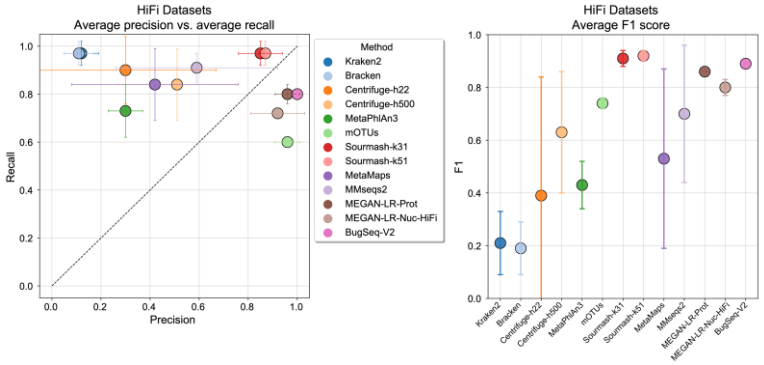
- Perform translation alignment of HiFi reads to a protein database using DIAMOND and summarize with MEGAN-LR, for the purpose of taxonomic and functional profiling.
- Provides access to NCBI and GTDB taxonomic annotations

Taxonomic-Profiling-Minimap-Megan



- Align HiFi reads to a nucleotide database using minimap2 and summarize with MEGAN-LR, for the purpose of taxonomic profiling
- Provides access to NCBI and GTDB taxonomic annotations

Taxonomic-Profiling-Sourmash



- obtain taxonomic profiles using `sourmash gather --> taxonomy` approach.
- Provides access to NCBI and GTDB taxonomic annotations, or you can build your own database.

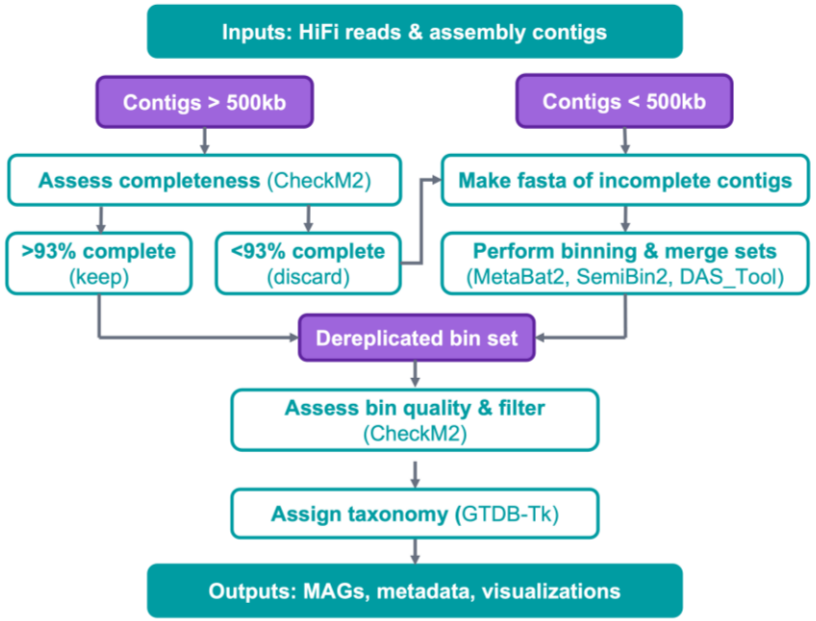
[1] Refer to https://github.com/PacificBiosciences/pb-metagenomics-tools for details about specific PB-metagenomics-tools pipelines.

Ampli-Fi data analysis recommendations for shotgun metagenomics

Use HiFi-MAG-Pipeline to obtain high-quality metagenome-assembled genomes (MAGs)

- Can perform metagenomic shotgun assembly directly with HiFi reads using third-party tools (e.g., hifiasm-meta, metaFlye or HiCanu) and evaluate & extract metagenome-assembled genomes using PacBio HiFi-MAG-Pipeline tool available on GitHub (see Portik *et al*.[1])

HiFi-MAG-Pipeline



- Streamlined HiFi-MAG-Pipeline workflow includes a custom "completeness-aware" strategy to identify and protect long & complete contigs
- Binning is performed with MetaBAT2 and SemiBin2; bin merging occurs with DAS_Tool, QC with CheckM2; and taxonomic assignments with GTDB-Tk
- Outputs include high-quality MAG sequences, summary figures, and associated metadata

- Contact PacBio Technical Support (support@pacb.com) or your local Field Applications Bioinformatics Support Scientist for additional information about data analysis recommendations

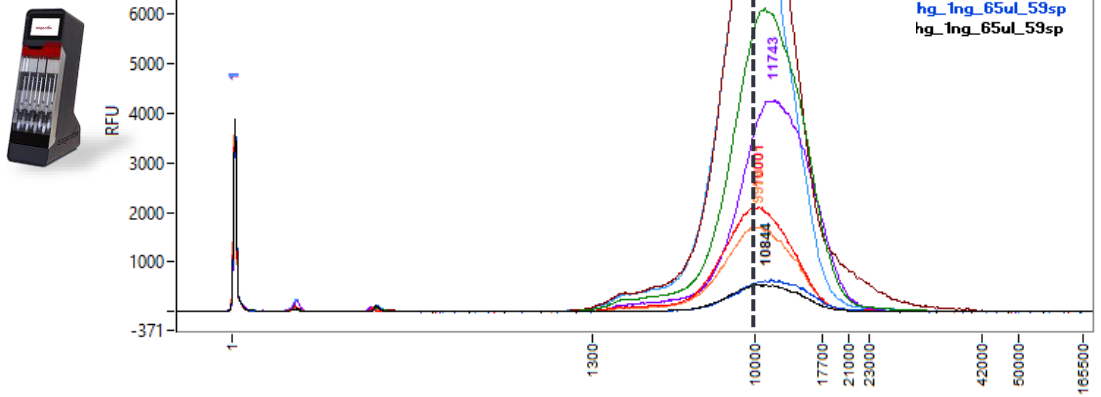
[1] Portik D.M. et al. (2022) Evaluation of taxonomic profiling methods for long-read shotgun metagenomic sequencing datasets. BMC Bioinformatics. 23:541. https://doi.org/10.1186/s12859-022-05103-0

Ampli-Fi library example sequencing performance data

Example Ampli-Fi library prep QC results for human gDNA samples[1]

Example DNA shearing and SMRTbell library construction yield results

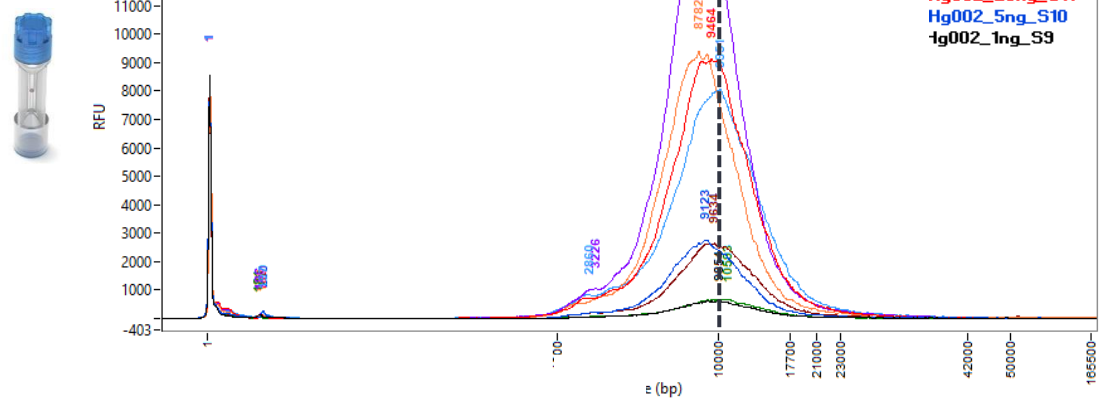
Megaruptor 3 system shearing



gDNA input (ng)	PCR cycles	Twist UDI barcode set	PCR yield (ng)	SMRTbell library yield	Post-ABC yield
1 ng	14 cycles	PlateA_bc26	1290	516 ng (40%)	410 ng (32%)
5 ng	12 cycles	PlateA_bc27	2020	828 ng (41%)	705 ng (35%)
20 ng	10 cycles	PlateA_bc29	1780	854 ng (48%)	585 ng (33%)
50 ng	8 cycles	PlateA_bc31	1660	747 ng (45%)	615 ng (37%)

Example Femto Pulse DNA sizing QC analysis results and Ampli-Fi library construction yield results for human genomic DNA samples sheared to ~10 kb target fragment mode size using a Megaruptor 3 system.

g-TUBE shearing



gDNA input (ng)	PCR cycles	Twist UDI barcode set	PCR yield (ng)	SMRTbell library yield	Post-ABC yield
1 ng	14 cycles	PlateA_bc77	1195	454 ng (38%)	404 ng (34%)
5 ng	12 cycles	PlateA_bc78	1510	604 ng (40%)	534 ng (35%)
20 ng	10 cycles	PlateA_bc79	2210	840 ng (38%)	744 ng (34%)
50 ng	8 cycles	PlateA_bc80	1340	456 ng (34%)	393 ng (29%)

Example Femto Pulse DNA sizing QC analysis results and Ampli-Fi library construction yield results for human genomic DNA samples sheared to ~10 kb target fragment mode size using a g-TUBE device.

[1] Example library prep QC results shown are for Ampli-Fi libraries generated from high-quality human HG002 DNA samples.

Example Ampli-Fi sequencing results for human gDNA samples[1]

Example HiFi sequencing metrics

Revio system with SPRQ chemistry

Sample	HiFi yield (Gb)	Pol RL (bp)	HiFi reads	HiFi RL (bp)	Mean QV	BQ≥Q30 (%)	*P1* (%)
MR_1ng	78.2	89,102	11.7 M	6,665	Q44	98	72
MR_5ng	80.4	85,987	12.3 M	6,521	Q43	97	75
MR_20ng	67.8	97,663	9.4 M	7,183	Q44	98	56
MR_50ng	84.5	94,644	11.1 M	7,604	Q43	97	67

Example HiFi sequencing results for Ampli-Fi libraries prepared using human gDNA sheared with a Megaruptor 3 system and run on a Revio system with SPRQ chemistry (120 pM OPLC).

Sample	HiFi yield (Gb)	Pol RL (bp)	HiFi reads	HiFi RL (bp)	Mean QV	BQ≥Q30 (%)	*P1* (%)
g-T_1ng	61.5	98,396	9.8 M	6,267	Q46	98	58
g-T_5ng	61.9	99,643	9.7 M	6,354	Q46	98	56
g-T_20ng	55.4	107,197	9.1 M	6,079	Q47	98	52
g-T_50ng	64.5	105,438	9.4 M	6,849	Q45	98	54

Example HiFi sequencing results for Ampli-Fi libraries prepared using human gDNA sheared with g-TUBEs and run on a Revio system with SPRQ chemistry (120 pM OPLC).

Vega system

Sample	HiFi yield (Gb)	Pol RL (bp)	HiFi reads	HiFi RL (bp)	Mean QV	BQ≥Q30 (%)	*Loading level* (%)
MR_1ng	64.9	95,508	9.6 M	6,777	Q42	97	59
MR_5ng	64.9	104,358	9.3 M	6,946	Q43	97	57
MR_20ng	59.2	113,624	8.6 M	6,852	Q44	98	43
MR_50ng	64.9	107,824	9.6 M	6,729	Q43	98	52

Example HiFi sequencing results for Ampli-Fi libraries prepared using human gDNA sheared with a Megaruptor 3 system and run on a Vega system (120 pM OPLC).

Sample	HiFi yield (Gb)	Pol RL (bp)	HiFi reads	HiFi RL (bp)	Mean QV	BQ≥Q30 (%)	*Loading level* (%)
g-T_1ng	63.9	99,318	9.0 M	7,070	Q42	97	50
g-T_5ng	62.0	79,705	10.3 M	6,002	Q42	97	62
g-T_20ng	64.7	100,310	10.2 M	6,332	Q43	97	55
g-T_50ng	64.7	99,617	9.0 M	7,190	Q41	97	58

Example HiFi sequencing results for Ampli-Fi libraries prepared using human gDNA sheared with g-TUBEs and run on a Vega system (120 pM OPLC).

HiFi data yield is dependent on input DNA quality → The more degraded the DNA, the lower the HiFi read length and base yield
Example yields shown in tables are based on high-quality human DNA samples prepared following best practices
→ Other sample types comprised of lower-quality DNA may show lower Ampli-Fi HiFi data yields

PacBio

[1] Example HiFi sequencing results shown are for 9 – 10 kb Ampli-Fi libraries generated from high-quality human HG002 DNA samples.

Technical documentation & applications support resources

Technical resources for Ampli-Fi library prep, sequencing & data analysis

DNA sample extraction literature & other resources

- Nanobind HMW DNA extraction Procedures & checklists [Link]
- Nanobind kit Guides & overviews [Link]
- Overview – Nanobind PanDNA HMW DNA extraction protocols (103-510-000)

SMRTbell library preparation literature & other resources

- Application brief – Comprehensive human genomic variant detection with HiFi long-read sequencing (102-326-626)
- Application brief – Metagenomic sequencing with HiFi reads (102-193-684)
- Application brief – Taxonomic and functional profiling with HiFi metagenomics (102-326-574)
- Brochure – Metagenomics solutions guide (102-326-512)
- Procedure & checklist – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (103-648-000)
- Technical note – Gel cassette size selection methods for HiFi libraries (102-326-503)
- Technical overview – Amplifying genomic DNA for SMRTbell library preparation and HiFi sequencing (106-645-000)

Publications

- McGinty, S.P. (2025) CiFi: Accurate long-read chromatin conformation capture with low-input requirements. bioRxiv. doi: https://doi.org/10.1101/2025.01.31.635566
- Bein, B. et al. (2024) Long-read sequencing and genome assembly of natural history collection samples and challenging specimens. bioRxiv. doi: https://doi.org/10.1101/2024.03.04.583385
- Männer L. et al. (2024) Chromosome-level genome assembly of the sacoglossan sea slug Elysia timida (Risso, 1818). bioRxiv. doi: https://doi.org/10.1101/2024.06.04.597355

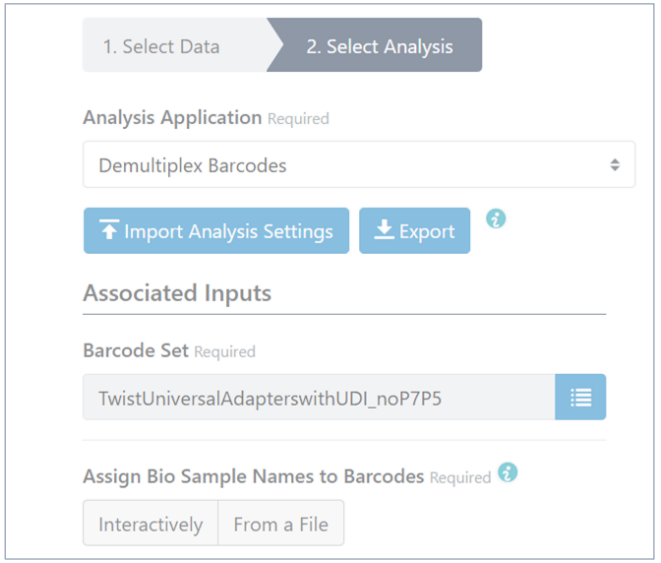
PacBio

Technical resources for Ampli-Fi library prep, sequencing & data analysis

Data analysis resources

- To demultiplex HiFi data sets generated for Ampli-Fi samples asymmetrically indexed with Twist Bioscience UDI PCR primers, download the files below from our Multiplexing Resources website and use them to set up a **Demultiplex Barcodes** utility job in SMRT Link:
  1. **`Amplifi_TwistUDIadapters_noP7P5.fasta`** barcode set file [Link]
     - Contains the following Twist (10-base pair) index sequences:
       - 16 UDI set, Twist 101307
       - 96 UDI set, Plate A, Twist 101308
       - 96 UDI set, Plate B, Twist 101309
       - 96 UDI set, Plate C, Twist 101310
       - 96 UDI set, Plate D, Twist 101311
     - Import this barcode set file into SMRT Link using the Data Management '**Import**' feature and select it when specifying the barcode set to use for the Demultiplex Barcodes utility job in SMRT Link
  2. **`Ampli-Fi_Barcoded_Sample_Name_File.csv`** bio sample name template file [Link]
     - Use this CSV template to assign bio sample names to pooled Ampli-Fi library samples indexed with Twist UDI PCR primers by editing and then saving the CSV file to your local computer
     - After saving the CSV file, upload it into SMRT Link using the '**From a File**' button to complete setting up the Demultiplex Barcodes utility job
- SMRT Link Cloud v25.2 user guide (103-654-800)
- SMRT Link v25.2 user guide (103-651-300)
- SMRT Link web services API use cases (103-653-100)
- SMRT Tools reference guide (103-653-200)

Barcode	Bio Sample Name
Plate_A_1_A01_F--Plate_A_1_A01_R	Ampli-Fi_library_01
Plate_A_2_B01_F--Plate_A_2_B01_R	Ampli-Fi_library_02
Plate_A_3_C01_F--Plate_A_3_C01_R	Ampli-Fi_library_03
Plate_A_4_D01_F--Plate_A_4_D01_R	Ampli-Fi_library_04



www.pacb.com