Technical overview – PureTarget repeat expansion panel library preparation using PureTarget kit

Sequel II and IIe systems ICS v11.0
Revio system ICS v13.1
SMRT Link v13.1

PN 103-418-100 Rev 01 | March 2024

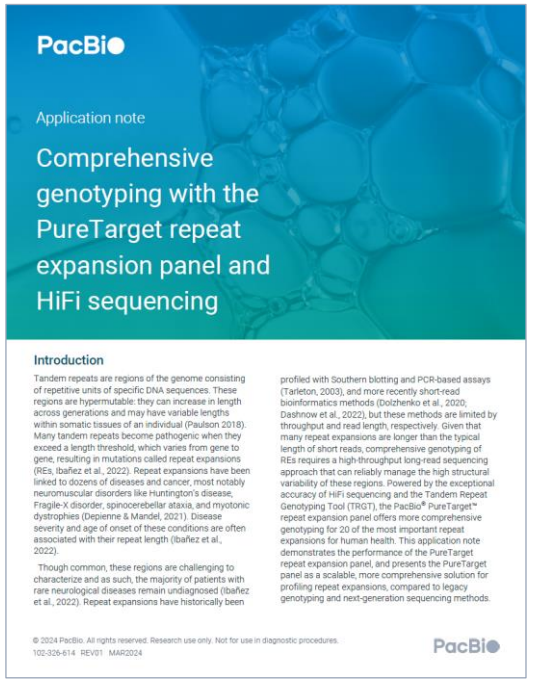
PureTarget repeat expansion panel library preparation using PureTarget kit

Technical Overview

1. PureTarget repeat expansion panel sequencing method overview
2. PureTarget repeat expansion panel library preparation workflow overview
3. PureTarget repeat expansion panel sequencing preparation workflow overview
4. PureTarget repeat expansion panel example sequencing performance data
5. PureTarget repeat expansion panel data analysis workflow overview
6. Technical documentation & applications support resources
7. Appendix

PureTarget repeat expansion panel library preparation using PureTarget kit: Getting started

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

Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing

Introduction

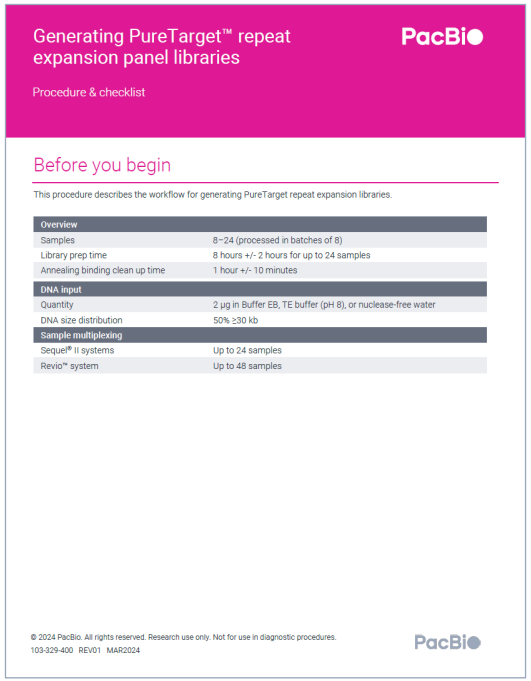
Tandem repeats are regions of the genome consisting of repetitive units of specific DNA sequences. These regions are hypermutable: they can increase in length across generations and may have variable lengths within somatic tissues of an individual (Paulson 2018). Many tandem repeats become pathogenic when they exceed a length threshold, which varies from gene to gene, resulting in mutations called repeat expansions (REs, Ibañez et al., 2022). Repeat expansions have been linked to dozens of diseases and cancer, most notably neuromuscular disorders like Huntington's disease, Fragile-X disorder, spinocerebellar ataxia, and myotonic dystrophies (Depienne & Mandel, 2021). Disease severity and age of onset of these conditions are often associated with their repeat length (Ibañez et al., 2022).

Though common, these regions are challenging to characterize and as such, the majority of patients with rare neurological diseases remain undiagnosed (Ibañez et al., 2022). Repeat expansions have historically been profiled with Southern blotting and PCR-based assays (Tarleton, 2003), and more recently short-read bioinformatics methods (Dolzhenko et al., 2020; Dashnow et al., 2022), but these methods are limited by throughput and read length, respectively. Given that many repeat expansions are longer than the typical length of short reads, comprehensive genotyping of REs requires a high-throughput long-read sequencing approach that can reliably manage the high structural variability of these regions. Powered by the exceptional accuracy of HiFi sequencing and the Tandem Repeat Genotyping Tool (TRGT), the PacBio® PureTarget™ repeat expansion panel offers more comprehensive genotyping for 20 of the most important repeat expansions for human health. This application note demonstrates the performance of the PureTarget repeat expansion panel, and presents the PureTarget panel as a scalable, more comprehensive solution for profiling repeat expansions, compared to legacy genotyping and next-generation sequencing methods.

102-326-614 REV01 MAR2024

Application note – Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing (102-326-614)

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


Generating PureTarget™ repeat expansion panel libraries

PacBio

Procedure & checklist

Before you begin

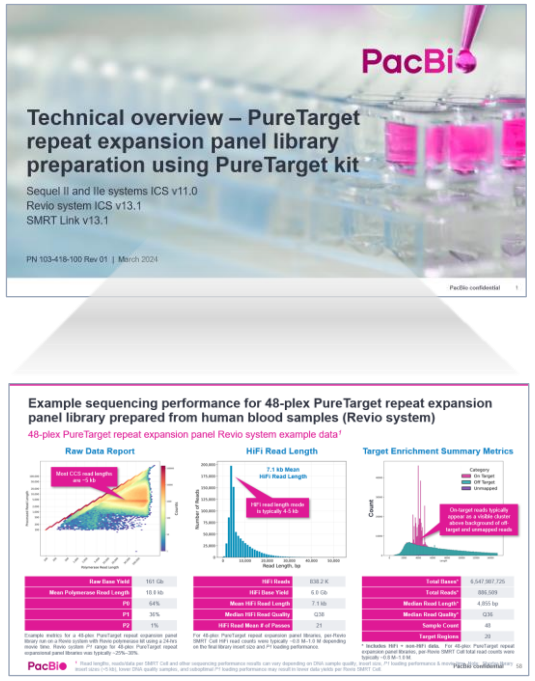
This procedure describes the workflow for generating PureTarget repeat expansion libraries.

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

103-329-400 REV01 MAR2024

Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400)

Technical documentation containing application-specific library preparation protocol details.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Technical overview – PureTarget repeat expansion panel library preparation using PureTarget kit

Sequel II and IIe systems ICS v11.0
Revio system ICS v13.1
SMRT Link v13.1

PN 103-418-100 Rev 01 | March 2024

Example sequencing performance for 48-plex PureTarget repeat expansion panel library prepared from human blood samples (Revio system)

48-plex PureTarget repeat expansion panel Revio system example data[1]

Raw Data Report

HiFi Read Length

Target Enrichment Summary Metrics

Technical Overview – PureTarget repeat expansion panel library preparation using PureTarget kit (103-418-100)

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

gDNA sample extraction & QC
(Nanobind kit)

2 µg DNA per sample
Genome quality number (30 kb) ≥5.0
Use Nanobind PanDNA kit for blood extraction

PureTarget library preparation
(PureTarget repeat expansion panel)

Use CRISPR/Cas9 & gRNAs to target repeats
SMRTbell prep kit 3.0 for library construction
Supports sample multiplexing up to 48-plex

SMRT sequencing
(Sequel II/IIe & Revio systems)

Perform ABC* and sequence PureTarget libraries on PacBio long-read systems

Data analysis
(SMRT Link)

Use SMRT Link PureTarget repeat expansion analysis application to characterize normal & expanded alleles in a 20-gene target panel

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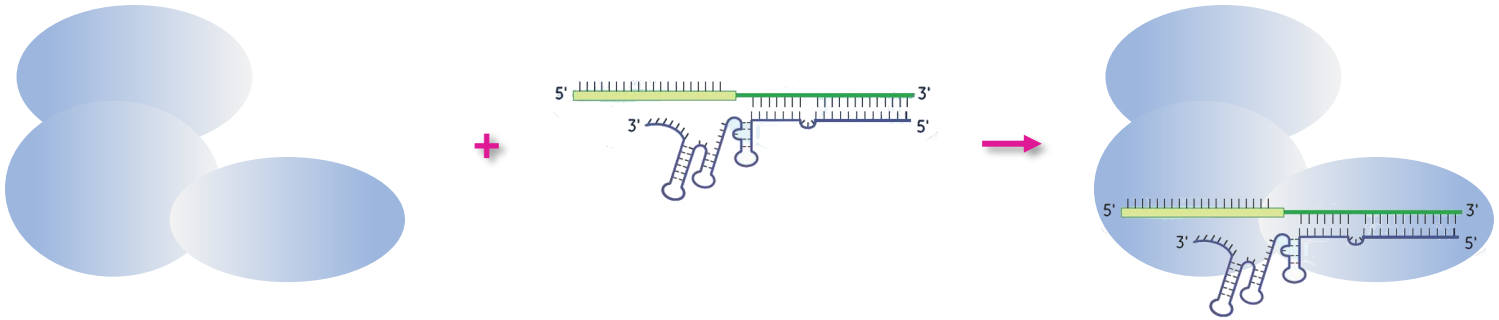
* ABC = Anneal primer / Bind polymerase / Clean up bound complex

PureTarget sequencing method overview

CRISPR-Cas9 technology overview

CRISPR-Cas9 system comprises a guide RNA (gRNA or sgRNA) and Cas9 nuclease, which together form a ribonucleoprotein (RNP) complex that can introduce a site-specific double-strand break in DNA[1,2]

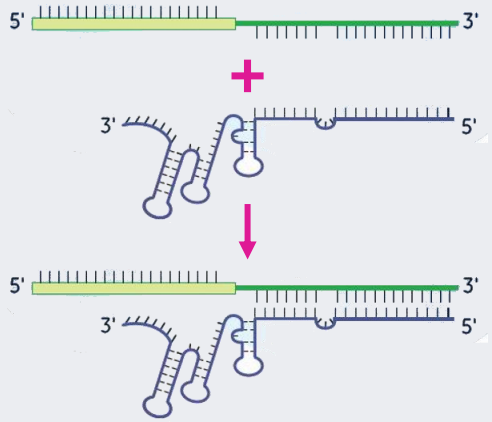


Cas9 nuclease | Guide RNA | Cas9-gRNA complex

Guide RNA configurations

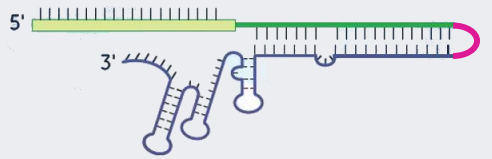
Two-component guide RNA (gRNA)

Annealed [crRNA : tracrRNA] complex



Single guide RNA (sgRNA)

Synthetic crRNA-tracrRNA single-molecule hybrid construct



sgRNA is used in PureTarget library prep workflow

1 *Image modified from:* https://horizondiscovery.com/en/applications/gene-editing

2 CRISPR (= *clustered regularly interspaced short palindromic repeats*) is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria that play a role in the anti-viral defense system of these organisms.

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CRISPR-Cas9 technology overview (cont.)

CRISPR-Cas9 system comprises a guide RNA (gRNA or sgRNA) and Cas9 nuclease, which together form a ribonucleoprotein (RNP) complex that can introduce a site-specific double-strand break in DNA[1,2]

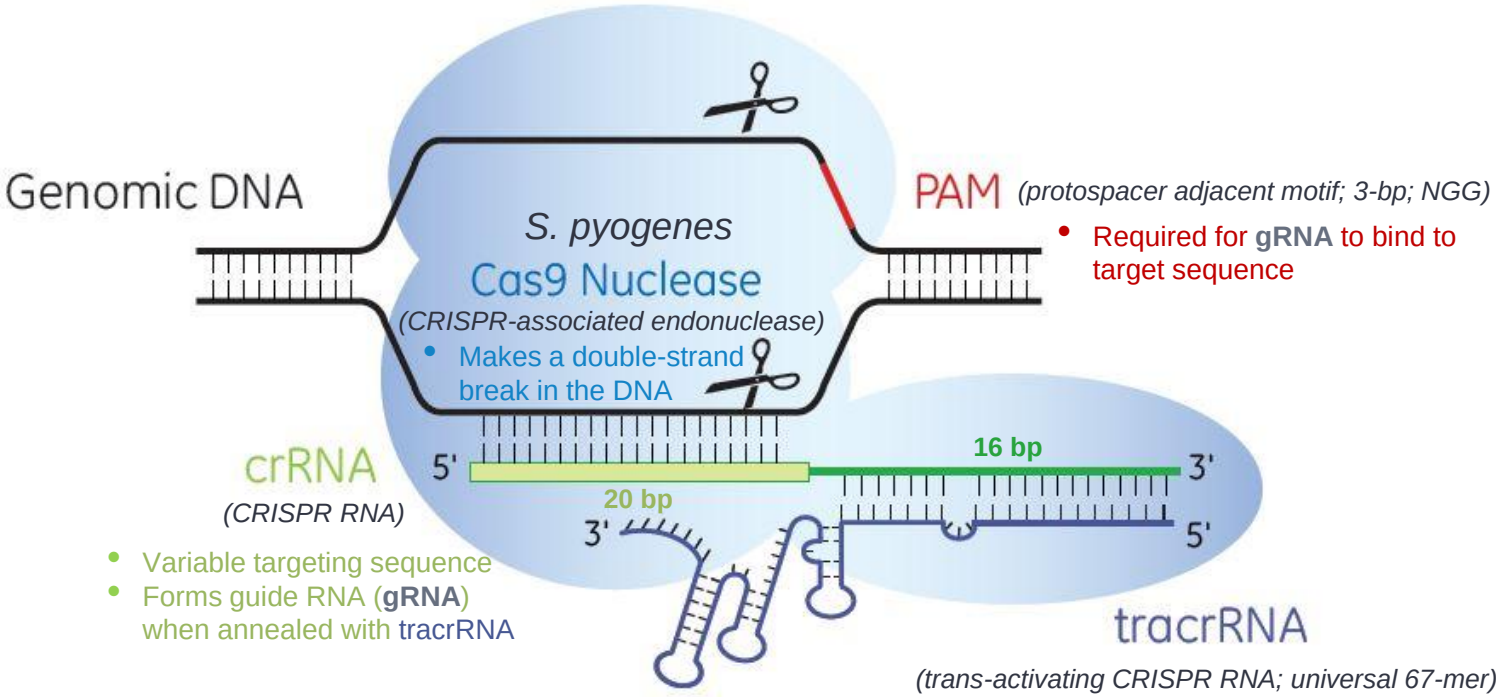
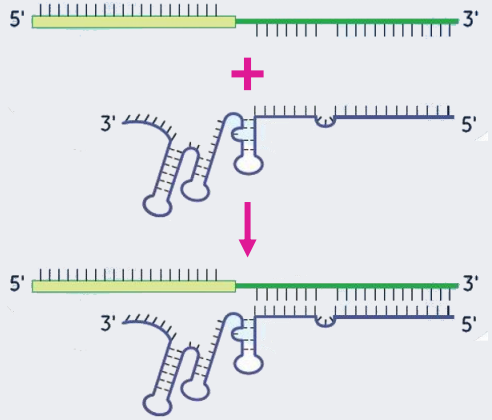


Illustration of **Cas9 nuclease** (blue), programmed by the **tracrRNA** (violet) : **crRNA** (olive) complex (= **guide RNA**) cutting both strands of genomic DNA 5' of the protospacer-adjacent motif (**PAM**) (red).

Guide RNA configurations

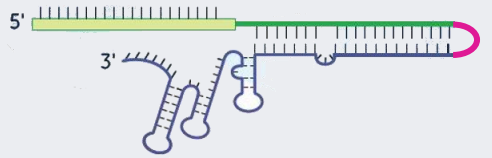
Two-component guide RNA (gRNA)

Annealed [crRNA : tracrRNA] complex



Single guide RNA (sgRNA)

Synthetic crRNA-tracrRNA single-molecule hybrid construct



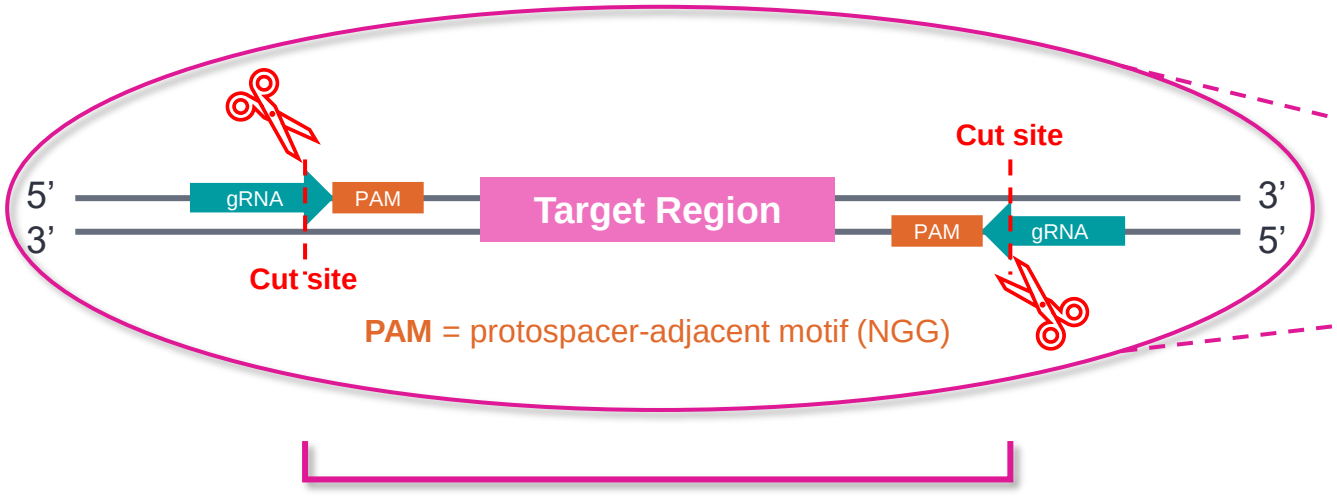
sgRNA is used in PureTarget library prep workflow

[1] *Image modified from:* https://horizondiscovery.com/en/applications/gene-editing

[2] CRISPR (= *clustered regularly interspaced short palindromic repeats*) is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria that play a role in the anti-viral defense system of these organisms.

How CRISPR-Cas9 is used in PureTarget native DNA library prep workflow

For PureTarget HiFi sequencing, CRISPR-Cas9 system is used to selectively enrich for regions of interest in a genomic sample prior to long-read sequencing of native DNA molecules[1]

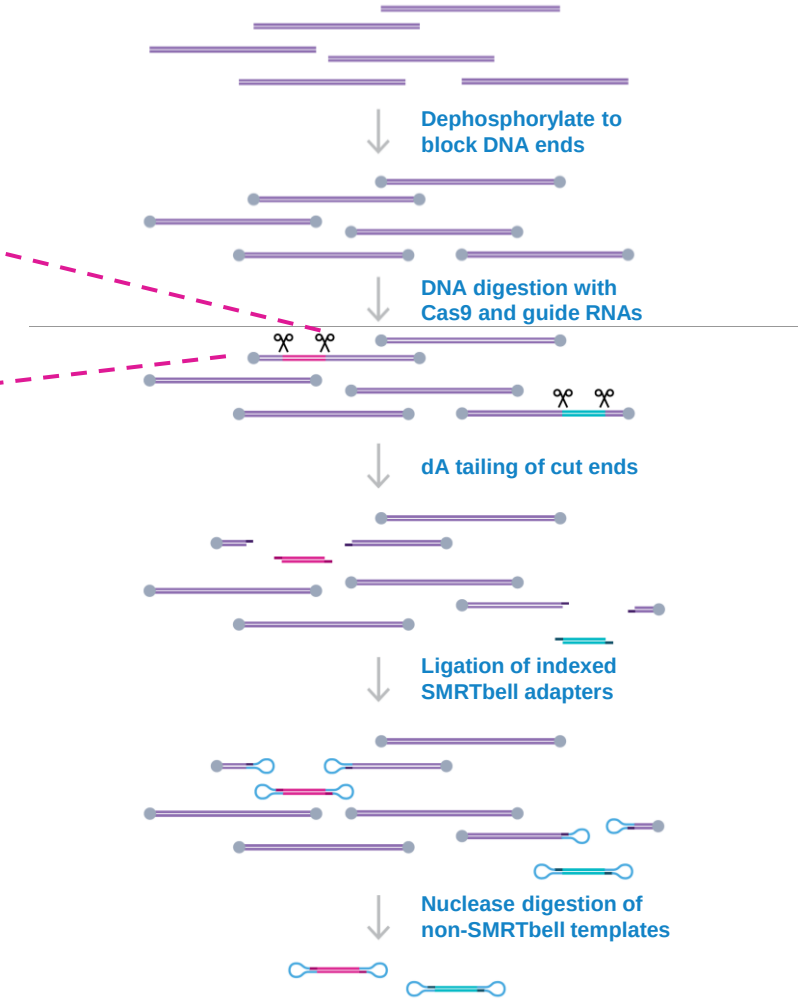


4 – 5 kb[2]

Excising a target region with two flanking gRNA-Cas9 complexes

- Cas9 nuclease, in close association with appropriate guide RNA (gRNA) oligonucleotides, identifies and then cleaves a specific recognition site on each side of the target region
- Different regions of interest can be simultaneously targeted in a single CRISPR-Cas9 digestion reaction by using multiple sets of gRNA pairs

Target enrichment with CRISPR-Cas9



[1] Tsai, Y. C., et al. (2022). Multiplex CRISPR/Cas9-Guided No-Amp targeted sequencing panel for spinocerebellar ataxia repeat expansions. In Genomic Structural Variants in Nervous System Disorders (pp. 95-120). New York, NY: Springer US.).

[2] **Note:** PureTarget guide RNAs are designed to cut a 5-kb region in the human reference genome; however, some DNA fragment sizes that are sequenced may be much larger with repeat expansion alleles.

PureTarget repeat expansion panel library preparation procedure description

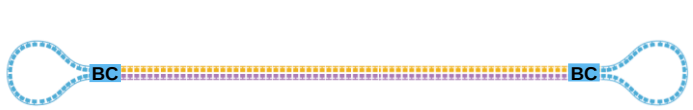
Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400) describes the workflow for generating PureTarget repeat expansion libraries using the **PureTarget kit** for sequencing on PacBio Sequel II/IIe & Revio systems

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

PureTarget repeat expansion panel kit bundle

103-390-400 (24 rxn)

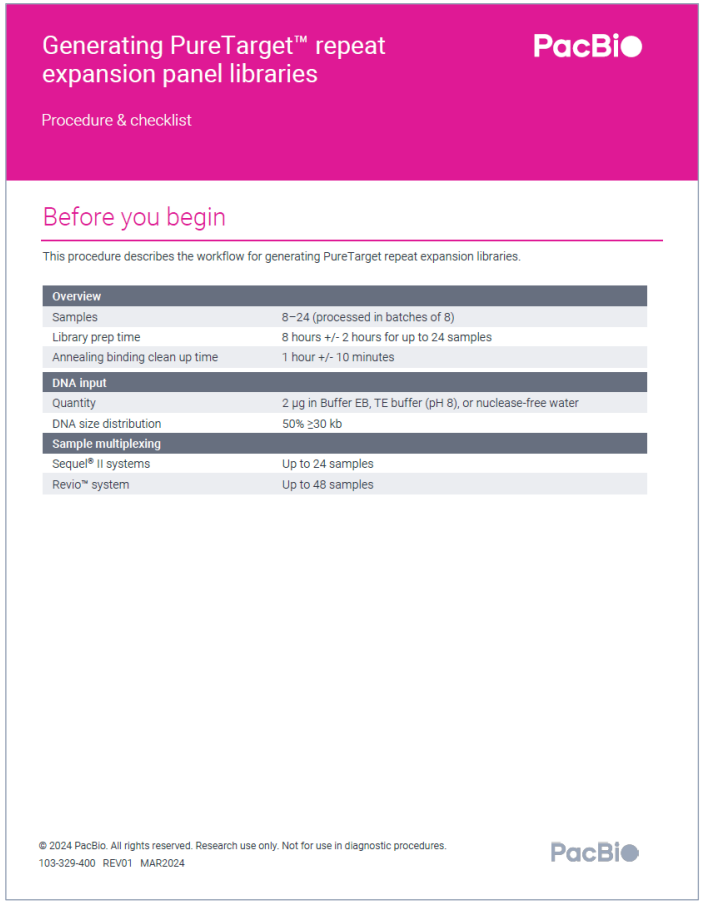
- Includes 20-gene panel and targeting reagents
- Also includes SMRTbell prep kit 3.0 reagents for library construction
- Supports 24 samples



PureTarget library template (~4 – 5 kb)

Contains indexed SMRTbell adapters[1]

- PureTarget library preparation procedure supports up to 48-plex sample multiplexing through use of 48 different SMRTbell indexed adapters[1]



Generating PureTarget™ repeat expansion panel libraries

PacBio

Procedure & checklist

Before you begin

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

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

103-329-400 REV01 MAR2024

PacBio

Note: Procedure 102-329-400 includes instructions for PureTarget repeat expansion panel **SMRTbell library construction workflow *and* sequencing preparation (ABC[2]) workflow**

[1] To enable sample multiplexing, SMRTbell adapter index plate 96A (102-009-200) must be purchased separately from PureTarget repeat expansion panel kit bundle (103-390-400).

[2] For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) (Step 11a for Revio systems or Step 11b for Sequel II/IIe systems) – Do not use SMRT Link Sample Setup.

PacBio

PureTarget repeat expansion panel kit bundle (103-390-400)[1,2]

PureTarget panel kit bundle supports amplification-free targeted sequencing library prep workflow

PureTarget repeat expansion targeting kit

- Includes reagents for PureTarget repeat expansion panel including Cas9 and guide RNAs for 20-target gene panel. Supports 24 samples.

PureTarget beads kit

- Includes reagents for performing SMRT boost bead washes in PureTarget library preparation workflows. Supports 3 bead washes (batch size = 8).

SMRTbell prep kit 3.0 (102-141-700)

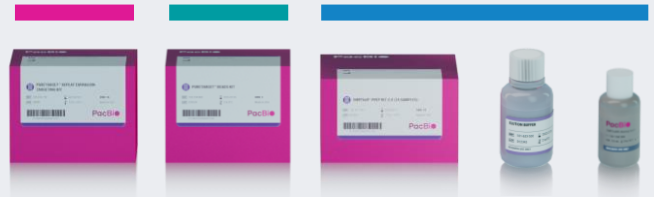
- Includes reagents for SMRTbell library preparation. Supports 24 samples.

PureTarget targeting kit components

Component		Description
1		**Cas9 buffer** • For Cas 9 digestion of gDNA
2		**Phosphatase** • For blocking gDNA fragment ends
3		**Cas9 nuclease** • For Cas 9 digestion of gDNA
4		**gRNA mix** • For Cas 9 digestion of gDNA
5		**dA tail buffer** • For A-tailing reaction
6		**dATP** • For A-tailing reaction
7		**Taq DNA polymerase** • For A-tailing reaction

PureTarget beads kit components

Component		Description
1		**SMRT boost beads** • For final cleanup of PureTarget repeat expansion panel libraries
2		**SMRT boost buffer** • For preparing SMRT boost beads for bead wash steps prior to use



PureTarget repeat expansion panel kit bundle
103-390-400 (24 rxn)

SMRTbell prep kit 3.0 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

[1] PureTarget repeat expansion targeting kit (103-234-700) and PureTarget beads kit (103-234-800) can only be purchased as part of the PureTarget repeat expansion panel kit bundle product (103-390-400). SMRTbell prep kit 3.0 (102-141-700) may be purchased as a stand-alone product.

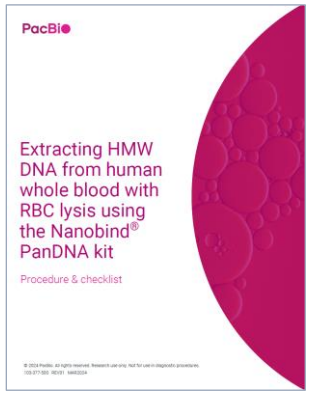
[2] PureTarget repeat expansion panel kit bundle (103-390-400) reagent volumes are optimized for batches of 8 and reagent volumes may be insufficient to support batching of fewer than 8. Specifically, a total of 3 SMRT boost bead washes are supported by reagent volumes in the kit.

Other recommended kits & consumables for PureTarget sample DNA extraction, sample multiplexing and SMRT sequencing

Ancillary kits must be purchased separately from PureTarget repeat expansion panel kit bundle (103-390-400)

HMW DNA extraction

Nanobind PanDNA kit
(103-260-000)



Procedure & checklist
(103-377-500)

- Supports 24 reactions for HMW DNA extraction from cells, bacteria, blood, tissue, insect, and plant nuclei
- Nanobind PanDNA kit is recommended for DNA extraction from cultured cells and human whole blood with RBC lysis for PureTarget repeat expansion panel applications[1]

Sample multiplexing

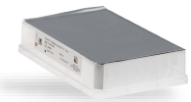
SMRTbell adapter index plate 96A
(102-009-200)

	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

- Contains 96 indexed SMRTbell adapters in plate format (1 sample per index)
- Plate includes indexes bc2001–bc2096

SMRT sequencing

Revio polymerase kit[2]
(102-817-600)

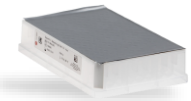


Revio sequencing plate
(4-rxn: 102-587-400)

Revio SMRT Cell tray
(4 cells: 102-202-200)

- Revio polymerase kit supports **12** Revio SMRT Cells for PureTarget repeat expansion panel samples

Sequel II binding kit 3.2 and cleanup beads
(102-333-300)



Sequel II sequencing kit 2.0
(4-rxn: 101-820-200)

Sequel II SMRT Cell 8M tray
(4 cells: 101-389-001)

- Sequel II binding kit supports **24** Sequel II SMRT Cells for PureTarget repeat expansion panel samples

[1] For genomic DNA extraction from blood, we recommend using the **red blood cell lysis protocol** described in ***Procedure & checklist – Extracting HMW DNA from human whole blood with RBC lysis using the Nanobind PanDNA kit*** (103-377-500) instead of extracting DNA directly from whole blood. For PureTarget repeat expansion panel applications, Nanobind CBB kit may also be used for HMW DNA extraction from cultured human cells.

[2] **Note:** PureTarget library preparation procedure (103-329-400) supports up to 48-plex sample multiplexing through use of 48 different SMRTbell indexed adapters.

PureTarget repeat expansion panel kit product specifications for supported sample types

PureTarget enables comprehensive characterization of repeat expansions at scale[1]

Parameter	Specification	Notes
Target gene panel size	20	• See ***Application note – Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing*** (102-326-614) for list of target genes included in PureTarget repeat expansion panel kit (103-390-400)
DNA input amount	2 μg DNA per sample	• 1 – 4 μg DNA per sample supported
DNA input quality	GQN30kb > 5	• 50% of mass of DNA molecules longer than 30 kb as measured on Femto Pulse (Agilent)
Target coverage	Mean target coverage: >200-fold	• Mean coverage for 2 μg of input DNA from supported sample types (Nanobind-extracted human blood and cell lines)[2] for unexpanded alleles
	Minimum target coverage: 50-fold	• Minimum coverage for 2 ug of input DNA from supported sample types (Nanobind-extracted human blood and cell lines)[2] for unexpanded alleles
Sample multiplexing	**Sequel II/IIe system:** Up to 24-plex	• Kit supports smaller batches in multiples of 8 samples
	Revio system: Up to 48-plex	• Kit supports smaller batches in multiples of 8 samples
Library insert size	4 – 5 kb	• Inserts with expanded alleles will be longer
Methylation	5mC in CpG sites detected	• Methylation probabilities for CpG sites are encoded in BAM output file
PureTarget library prep protocol	***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400)	• Requires PureTarget repeat expansion panel kit bundle (103-390-400)
SMRT Link data analysis workflow	PureTarget repeat expansion analysis application	• SMRT Link v13.1+ required

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1 See ***Brochure – Comprehensive characterization of repeat expansions with PureTarget*** (102-326-609)

2 Optimal performance is obtained using PacBio Nanobind extraction kits with **human blood or cell line samples** as the officially supported sample type for the PureTarget kit.

PureTarget kit supported use cases and experimental design considerations

PureTarget kit offers a gene panel for 20 of the most important repeat expansions for human health

Disease	PureTarget gene panel targets
Spinocerebellar ataxia	*ATN1, ATXN1, ATXN2, ATXN3, ATXN7, ATXN8, ATXN10, CACNA1A, PPP2R2B, TBP*
CANVAS disease	*RFC1*
Fragile-X Disease	*FMR1*
Friedrich's ataxia	*FXN*
Huntington's Disease	*HTT*
Myotonic Dystrophy	*DMPK, CNBP*
Fuchs Endothelial Corneal Dystrophy	*TCF4*
ALS/FTD	*C9ORF72*
Spinal bulbar muscular atrophy	*AR*

PureTarget repeat expansion panel enables genotyping of critical pathogenic repeat expansion loci at scale

- PureTarget kit includes a panel of 20 repeat expansion loci with the panel capturing ~2 kb upstream and downstream of the repeat (total panel size is ~100 kb)
- For normal alleles, resulting sequences are 4–5 kb in length but reads for expanded alleles may be longer
- Sequencing results for samples with large expansions indicate that it is possible to span repeats up to 35 kb in length in a single read

Note about using custom panel designs[1]

- Custom PureTarget panels are not officially supported by PacBio, but we can offer guidance to users interested in designing and optimizing their own panels
- In all cases, we recommend first demonstrating success on the PureTarget repeat expansion panel using supported sample types before adding new guide RNAs or testing a custom set of guides
- PureTarget repeat expansion panel is in total ~100 kb in length so performance in panels that are much smaller or much larger is unknown and may require optimization of wet lab protocol or reagents

[1] See ***Application note – Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing*** (102-326-614) for guidance surrounding support for custom PureTarget repeat expansion panels. **Note:** PureTarget repeat expansional panel kit does not support analysis of non-human samples as the guide RNAs used in the panel are designed again the human genome.

PureTarget library preparation workflow overview

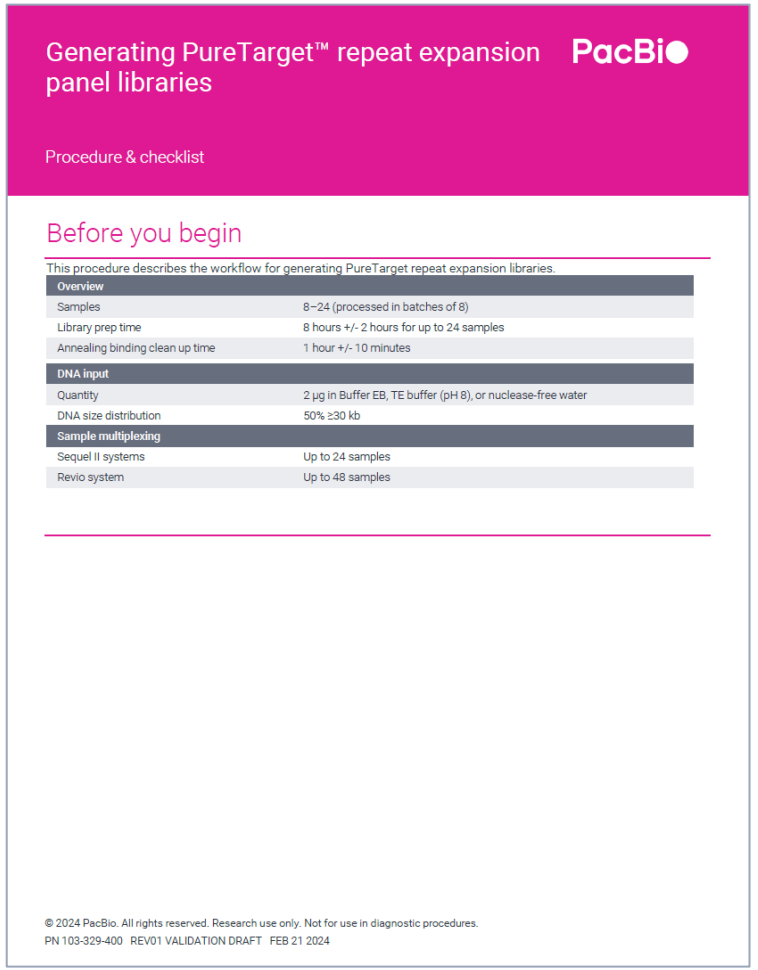
Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400)

Procedure & checklist 103-329-400 describes the workflow for generating PureTarget repeat expansion libraries using the **PureTarget kit** for sequencing on PacBio Sequel II/IIe & Revio systems

Procedure & checklist contents

1. DNA input QC recommendations and general best practices for reagent & sample handling.
2. Instructions for Cas9/gRNA complex formation.
3. Enzymatic workflow steps for performing targeted Cas9 digestion of input genomic DNA samples.
4. Enzymatic workflow steps for PureTarget SMRTbell library construction from Cas9-digested gDNA samples.
5. Workflow steps for final cleanup of PureTarget SMRTbell libraries using SMRT boost bead wash and SMRTbell cleanup beads
6. Workflow steps for sample setup ABC[1] to prepare samples for sequencing

Note: Procedure 102-329-400 includes instructions for PureTarget repeat expansion panel **SMRTbell library construction workflow *and* sequencing preparation (ABC[1]) workflow**



Generating PureTarget™ repeat expansion panel libraries

PacBio

Procedure & checklist

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DNA size distribution	50% ≥30 kb
Sample multiplexing	
Sequel II systems	Up to 24 samples
Revio system	Up to 48 samples

PN 103-329-400 REV01 VALIDATION DRAFT FEB 21 2024

PacBio Documentation (103-329-400)

[1] For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) (Step 11a for Revio systems or Step 11b for Sequel II/IIe systems) – Do not use SMRT Link Sample Setup.

PureTarget library construction workflow overview

Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400)

	Cleanup	DNA QC	Walk-away time[1]
1 Input DNA quality control	N/A	Qubit DNA HS assay Femto Pulse gDNA 165 kb kit	N/A
2 Dephosphorylation	N/A	N/A	15 min
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3 Cas9-gRNA complex formation	N/A	N/A	15 min
4 Cas-9 digestion & cleanup	1.0X SMRTbell cleanup beads	Qubit dsDNA HS assay	1.5 hrs
Safe stop			
5 dA tail	N/A	N/A	30 min
6 Adapter ligation & cleanup	1.0X SMRTbell cleanup beads	Qubit dsDNA HS assay	1 hr
Safe stop			
7 Nuclease Treatment	N/A	N/A	1 hr
8 Sample pooling & cleanup	1.0X SMRTbell cleanup beads	N/A	0.5 hrs
Safe stop			
9 SMRT boost wash & cleanup	1.0X SMRTbell cleanup beads	N/A	45 min
10 Concentrate samples for ABC	1.0X SMRTbell cleanup beads	Qubit dsDNA HS assay	15 min
Safe stop			

<6 hrs[1]

Including hands-on time, this library prep procedure takes **8 hrs (+/- 2 hrs) to process up to 24 samples**

[1] **Excludes** hands-on time for setting up enzymatic reaction steps and additional time required to perform DNA sizing QC and DNA concentration QC.

General best practices recommendations for preparing PureTarget libraries

Supported input sample types and DNA extraction methods

- It is recommended that users obtain high-quality genomic DNA with Nanobind extraction kits[1] from PacBio
- Officially supported sample types include:
  - Whole blood extracted using red blood cell (RBC) lysis method and the Nanobind PanDNA kit
  - Peripheral blood mononuclear cells (PBMCs) extracted with Nanobind CBB kit
  - Human cell lines extracted with Nanobind PanDNA or Nanobind CBB kit.
- When using sample types and extraction methods other than the above, we recommend users:[3]
  - First, demonstrate success using supported sample types, starting with an 8-plex and increasing sample quantity thereafter
  - Introduce new sample types or extraction methods in limited numbers, for example, 3 or fewer new sample types in an 8-plex of otherwise controls
  - See table at right for more information about samples that are officially supported, have been tested, or are not supported

Nanobind PanDNA kit (103-260-000)

Supports 24 reactions per kit and includes:

- Nanobind PanDNA kit RT (103-260-300)
- Nanobind PanDNA kit 4C (103-260-400)

Human sample type	Extraction method	Category
Whole blood	Nanobind PanDNA kit (103-260-000); extracted using RBC-lysis method	Supported
Peripheral blood mononuclear cells (PBMC)	Nanobind CBB kit (102-301-900)	
Commercial lymphoblastoid cell lines	Nanobind CBB kit (102-301-900) Nanobind HT CBB kit – automated (102-762-700)	
Skeletal muscle Brain tissue Myoblasts	Nanobind PanDNA kit (103-260-000)	Tested in low plex
Whole blood	FlexiGene DNA Whole Blood Kit– automated (AutoGen AGKT-FG-640)	Tested in high plex
Whole blood	Genomic-Tip (Qiagen)	
Whole blood	QIAsymphony (Qiagen)	
Whole blood Corneal endothelial (CEC) cell culture	SP Blood and Cell Culture DNA Isolation Kit (Bionano 80042)	Tested
Whole blood	Monarch HMW DNA Extraction Kit for Cells & Blood New England Biolabs T3050S/T3050L	
Whole blood	Gentra Puregene Blood Kit (Qiagen 158467 / 158389)	
Fibroblasts	Genomic-Tip (Qiagen)	
Whole blood	chemagic DNA blood kit (Revity)	Not recommended

Guidance on sample types and extraction methods. Low plex means that fewer than 8 samples extracted with this method were pooled with other sample extraction types and run on a SMRT Cell at 8-plex or higher. High plex means 8 or more samples extracted with the method were pooled and run on a SMRT Cell.

[1] Refer to PacBio Documentation site for a list of supported Nanobind HMW DNA extraction protocols.
[3] See ***Brochure – Nanobind PanDNA kit*** (102-326-604).
[3] See ***Application note – Comprehensive genotyping with PureTarget repeat expansion panel and HiFi sequencing*** (102-326-614)

General best practices recommendations for preparing PureTarget libraries

Reagent and sample handling

- Room temperature is defined as any temperature in the range of 18-23°C for this protocol
- Mix all reagents well prior to use
- Quick spin all reagents in microcentrifuge to collect liquid at bottom prior to use
- Keep all temperature-sensitive reagents on ice
- Bring SMRTbell cleanup beads, SMRT boost beads, and Qubit reagents to room temperature for 30 minutes prior to use
- Pipette-mix all reactions by pipetting up and down 10 times. Use full-volume pipette mixing of all reactions to ensure thorough mixing of all reaction components
- Samples can be stored at -20°C at all safe stopping points listed in the protocol

Temperature-sensitive reagents

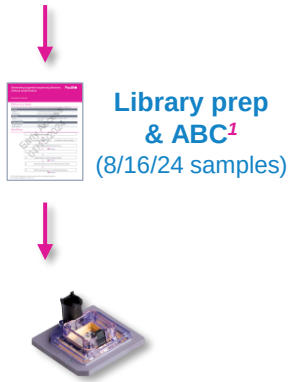
Protocol step	Reagent
Dephosphorylation	Phosphatase
Cas9 digestion	Cas9 Nuclease
Cas9 digestion	gRNA mix
dA Tailing	*Taq* DNA Polymerase
dA Tailing	dATP
Adapter ligation	SMRTbell adapter
Adapter ligation	Ligation mix
Adapter ligation	Ligation enhancer
Nuclease treatment	Nuclease mix
Reaction cleanups	SMRTbell cleanup beads

General best practices recommendations for preparing PureTarget libraries (cont.)

Multiplexed sample processing

Sequel II and Sequel IIe systems

8, 16 or 24 samples

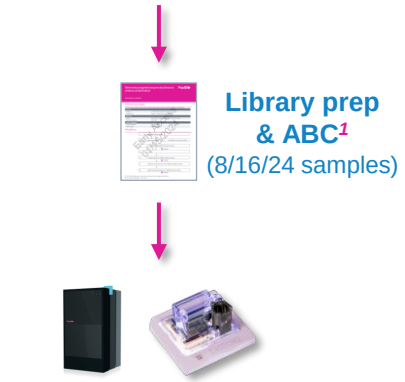


Sequence pooled samples
on one Sequel II SMRT Cell

- Up to 24 samples can be barcoded and sequenced per Sequel II SMRT Cell
- Multiplexed samples should be processed in batches of 8/16/24 samples
  - Follow protocol to process all samples in a **single** workflow

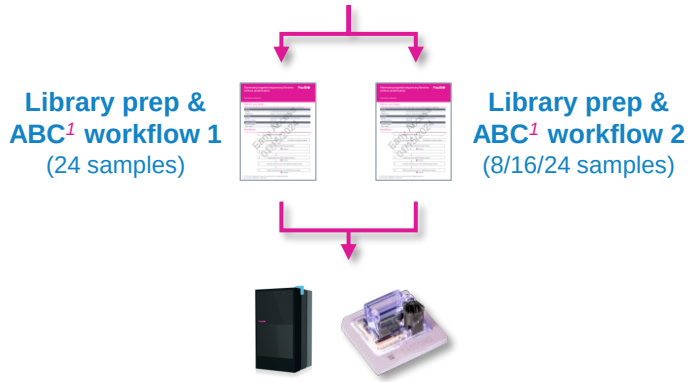
Revio systems

8, 16 or 24 samples



Sequence pooled samples
on one Revio SMRT Cell

32, 40 or 48 samples



Sequence pooled samples
on one Revio SMRT Cell

- Up to 48 samples can be barcoded and sequenced per Revio SMRT Cell
- Multiplexed samples should be processed in batches of 8/16/24/32/40/48 samples
- For Revio runs with 8/16/24 samples, follow protocol to process all samples in a **single** workflow
- For Revio runs with 32/40/48 samples, process **two** workflows in parallel:
  - **Workflow 1:** Process 24 samples following protocol; and
  - **Workflow 2:** Process an additional 8, 16 or 24 samples following protocol

[1] For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) – Do not use SMRT Link Sample Setup.

Input DNA quality control

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

1 Input DNA quality control

2 Dephosphorylation

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3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

Safe stop

5 dA tail

6 Adapter ligation & cleanup

Safe stop

7 Nuclease Treatment

8 Sample pooling & cleanup

Safe stop

9 SMRT boost wash & cleanup

10 Concentrate samples for ABC

Safe stop

PacBio

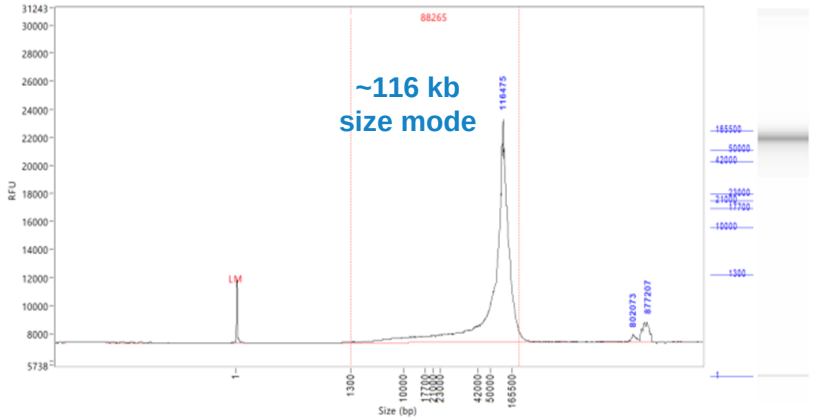
1. Input DNA quality control and dilution

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to room temperature.
	1.2	Pulse vortex and/or pipette-mix each sample 5 times to homogenize the DNA in solution. For viscous input DNA, it is important to homogenize the extracted DNA prior to start of the protocol. To homogenize the DNA, pulse-vortex 5 times and/or pipette-mix full sample volume 5 times, up and down with standard (not wide bore) tips. These steps will maintain HMW of your DNA but will improve accuracy of quantification and subsequent handling.
	1.3	Quick-spin each sample to collect liquid.
	1.4	Take a 1 µL aliquot from each sample and dilute with 9 µL of elution buffer or water.
	1.5	Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.
	1.6	Dilute each aliquot to 250 pg/µL in Femto Pulse dilution buffer based on the Qubit reading.
	1.7	Measure DNA size distribution with a Femto Pulse system using the gDNA 165 kb analysis kit.
	1.8	Aliquot or bring 1–4 µg DNA to a final volume of 67 µL per sample with nuclease-free water and transfer to an 8 tube PCR strip.

SAFE STOPPING POINT - Store at 4°C

- For **viscous** input DNA, it is important to homogenize the extracted DNA prior to starting the protocol

- Protocol requires high-quality, high molecular weight (HMW) human gDNA with ≥50% of the mass of DNA in molecules of length ≥30 kb, or Genome quality number (GQN) at 30 kb of ≥5 based on Agilent Femto Pulse system.
- Recommended input DNA amount is 2 µg per sample to ensure sufficient gene copies to load and maximize sequencing coverage. This protocol supports 1-4 µg input DNA per sample.[1]



Example Femto Pulse genomic DNA sizing QC analysis results for high-molecular weight genomic DNA extracted from a human whole blood sample using Nanobind PanDNA kit.

[1] The standard DNA mass recommended for PureTarget libraries is **2 µg**. Users who wish to increase coverage of a sample may use up to 4 µg of DNA in library prep. If higher coverage is needed, we recommend multiple preps of 4 µg for the sample and combining the data during analysis.

Dephosphorylation

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

1 Input DNA quality control

2 Dephosphorylation

3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

Safe stop

5 dA tail

6 Adapter ligation & cleanup

Safe stop

7 Nuclease Treatment

8 Sample pooling & cleanup

Safe stop

9 SMRT boost wash & cleanup

10 Concentrate samples for ABC

Safe stop

2. Dephosphorylation

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

Reaction Mix 1 (RM1)

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

	2.2	Pipette-mix RM1 up and down 10 times (do not vortex). Pipette-mix full volume.
	2.3	Quick-spin RM1 in a microcentrifuge to collect liquid.
	2.4	Add 13 µL of the RM1 to each sample from step 1.8 for total reaction volume of 80 µL.
	2.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
	2.6	Quick-spin in a microcentrifuge to collect liquid.
	2.7	Run the dephosphorylation thermocycler program. Set the lid temperature to 95°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

Step	Time	Temperature
1	10 min	37°C
2	3 min	80°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 (8-plex, 16-plex or 24-plex), plus 15% overage according to table

- Run **dephosphorylation** thermocycler program
- Set the lid temperature to 95°C (if lid temp. is not programmable, leave at 95–105°C)

Cas9-gRNA complex formation

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

1 Input DNA quality control
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2 Dephosphorylation
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3 Cas9-gRNA complex formation
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4 Cas-9 digestion & cleanup
↓ Safe stop
5 dA tail
↓
6 Adapter ligation & cleanup
↓ Safe stop
7 Nuclease Treatment
↓
8 Sample pooling & cleanup
↓ Safe stop
9 SMRT boost wash & cleanup
↓
10 Concentrate samples for ABC
Safe stop

3. Cas9-gRNA complex formation

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

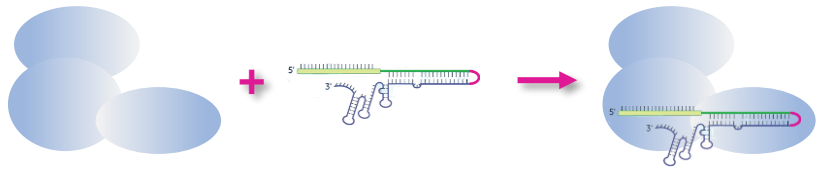
Reaction Mix 2 (RM2)

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

3.2	Pipette-mix RM2 up and down 10 times (do not vortex). Pipette-mix full volume.
3.3	Ensure RM2 is fully mixed and quick-spin in a microcentrifuge to collect liquid. Proceed to incubating the RM2 Master Mix using thermocycler program below. The full master mix volume for 8, 16, or 24 samples should be incubated in one PCR tube.
3.4	Run the Cas9 gRNA complex formation thermocycler program. Set the lid temperature to 75°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

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

3.5	Proceed to the next step of the protocol.



Cas9 nuclease | Guide RNA mix | Cas9-gRNA complex

Perform Cas9-gRNA complex formation during genomic DNA dephosphorylation reaction incubation

- 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 (8-plex, 16-plex or 24-plex), plus 25% overage according to table

- Run **Cas9 gRNA complex formation** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

Cas9 digestion & cleanup

This step enables digestion of double-stranded DNA at targeted regions using Master Mix RM2 prepared in Step 3

1 Input DNA quality control

2 Dephosphorylation

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3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

Safe stop

5 dA tail

6 Adapter ligation & cleanup

Safe stop

7 Nuclease Treatment

8 Sample pooling & cleanup

Safe stop

9 SMRT boost wash & cleanup

10 Concentrate samples for ABC

Safe stop

4.1 Cas9 digestion

✔	Step	Instructions
	4.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 15% overage according to the table below. RM2 is Cas9 gRNA complex from step 3 after thermocycler program.

Reaction Mix 3 (RM3)

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

- 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 (8-plex, 16-plex or 24-plex), plus 15% overage according to table

4.7 Run the Cas-9 digestion thermocycler program.

Set the lid temperature to 75°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

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

- Run **Cas-9 digestion** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

4.8 Quick-spin in a microcentrifuge to collect liquid.

4.9 1X SMRTbell bead cleanup

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

4.10 Pipette-mix the beads slowly 8–10x until evenly distributed. Bead clumping may occur and is not a concern, however, avoid over-pipetting as it may cause DNA/bead mixture to stick to the pipette tip.

- **Optional QC step after completing 1X SMRTbell bead cleanup:** Measure DNA concentration using Qubit 1X dsDNA HS kit
- Expected recovery is **~50-100%** per sample relative to starting input DNA mass

dA tail

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

1 Input DNA quality control

2 Dephosphorylation

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3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

Safe stop

5 dA tail

6 Adapter ligation & cleanup

Safe stop

7 Nuclease Treatment

8 Sample pooling & cleanup

Safe stop

9 SMRT boost wash & cleanup

10 Concentrate samples for ABC

Safe stop

4. dA tail

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

Reaction Mix 4 (RM4)

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

5.2	Pipette-mix RM4 up and down 10 times (do not vortex). Pipette-mix full volume.
5.3	Quick-spin RM4 in a microcentrifuge to collect liquid.
5.4	Add 10 µL of the RM4 to 40 µL of each sample for a total reaction volume of 50 µL.
5.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
5.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
5.7	Run the dA-tail thermocycler program. Set the lid temperature to 75°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

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

5.8	Proceed to the next step of the protocol.

- 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 (8-plex, 16-plex or 24-plex), plus 15% overage according to table

- Run **dA-tail** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

Adapter ligation & cleanup

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

1 Input DNA quality control

2 Dephosphorylation

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3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

Safe stop

5 dA tail

6 Adapter ligation & cleanup

Safe stop

7 Nuclease Treatment

8 Sample pooling & cleanup

Safe stop

9 SMRT boost wash & cleanup

10 Concentrate samples for ABC

Safe stop

6. Adapter ligation

✔	Step	Instructions
	6.1	To a PCR strip with 50 µL DNA from Step 5, add 4 µL SMRTbell barcoded adapter 3.0 to each sample. Tap-mix or pipette up and down 10 times (do not vortex).
	6.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 15% overage according to the table below. Aspirate and dispense viscous ligation mix slowly to reduce liquid sticking to the exterior and interior wall of the pipette tip.

Reaction Mix 5 (RM5)

✔	Reagent	Component	Volume per sample	Volume Per 8-plex With 15% overage	Volume Per 16-plex With 15% overage	Volume Per 24-plex With 15% overage
		NF water	2 µL	18.4 µL	36.8 µL	55.2 µL
	Purple	Repair buffer	8 µL	73.6 µL	147.2 µL	220.8 µL
	Yellow	Ligation mix	30 µL	276 µL	552 µL	828 µL
	Red	Ligation enhancer	1 µL	9.2 µL	18.4 µL	27.6 µL
		Total volume	41 µL	377.2 µL	754.4 µL	1131.6 µL

6.8	Run the adapter ligation thermocycler program. Set the lid temperature to 75°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.	

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

- Add 4 µL **SMRTbell adapter index plate 96A** to each sample and then tap-mix or pipette up and down 10 times (do not vortex)

- 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 (8-plex, 16-plex or 24-plex), plus 15% overage according to table

- Run **adapter ligation** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

1X SMRTbell bead cleanup

✔	Step	Instructions
	6.9	Add 95 µL of resuspended, room-temperature SMRTbell cleanup beads to
	6.10	Pipette-mix the beads 10 times until evenly distributed.

- **Optional QC step after completing 1X SMRTbell bead cleanup:** Measure DNA concentration using Qubit 1X dsDNA HS kit
- Expected recovery is **~50-100%** per sample relative to starting input DNA mass

Nuclease treatment

Nuclease treatment step removes unligated DNA fragments[1] and also removes leftover SMRTbell adapters from the sample

1 Input DNA quality control

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2 Dephosphorylation

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3 Cas9-gRNA complex formation

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4 Cas-9 digestion & cleanup

↓ ○ Safe stop

5 dA tail

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6 Adapter ligation & cleanup

↓ ○ Safe stop

7 Nuclease Treatment

↓

8 Sample pooling & cleanup

↓ ○ Safe stop

9 SMRT boost wash & cleanup

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10 Concentrate samples for ABC

○ Safe stop

7. Nuclease treatment

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

Reaction Mix 6 (RM6)

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

	7.2	Pipette-mix RM6 up and down 10 times (do not vortex). Pipette-mix full volume.
	7.3	Quick-spin RM6 in a microcentrifuge to collect liquid.
	7.4	Add 10 µL of RM6 to 40 µL of each sample. The total volume should equal 50 µL.
	7.5	Pipette-mix each sample up and down 10 times. Pipette-mix full volume.
	7.6	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	7.7	Run the nuclease treatment thermocycler program. Set the lid temperature to 75°C. If the lid temperature is not programmable, it is acceptable to leave at 95–105°C.

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

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

- 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 (8-plex, 16-plex or 24-plex), plus 15% overage according to table

- Run **nuclease treatment** thermocycler program
- Set the lid temperature to 75°C (if lid temp. is not programmable, leave at 95–105°C)

[1] Unligated DNA fragments comprise the majority of DNA fragments present prior to this step.

Sample pooling & cleanup

After nuclease treatment of individual samples, pool SMRTbell templates in units of 8 samples and perform cleanup with 1x SMRTbell cleanup beads

1 Input DNA quality control

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2 Dephosphorylation

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3 Cas9-gRNA complex formation

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4 Cas-9 digestion & cleanup

↓ ○ Safe stop

5 dA tail

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6 Adapter ligation & cleanup

↓ ○ Safe stop

7 Nuclease Treatment

↓

8 Sample pooling & cleanup

↓ ○ Safe stop

9 SMRT boost wash & cleanup

↓

10 Concentrate samples for ABC

○ Safe stop

8. Pooling samples for multiplex and 1X SMRTbell bead cleanup

✔	Step	Instructions for pooling, bead binding, washing, and sample elution
	8.1	In a 1.5 mL DNA LoBind tube combine nuclease treated libraries from step 7.7 in groups of 8 (8 x 50 µL) for a final sample volume of 400 µL.
	8.2	Add 400 µL of SMRTbell cleanup beads to each pooled nuclease treated sample.
	8.3	Pipette-mix up and down 10 times until the beads are evenly distributed. Pipette-mix full volume.
	8.4	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
	8.5	Leave at room temperature for 10 minutes to allow DNA to bind beads.
	8.6	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	8.8	Slowly dispense **1 mL** of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
	8.9	Repeat the previous step.
	8.10	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	8.11	Remove the tube strip from the magnetic rack. Immediately add 100 µL of elution buffer to each tube and resuspend the beads by pipetting up and down 10 times.
	8.13	Leave at room temperature for 5 minutes to elute DNA.
	8.14	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	8.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a 1.5 mL DNA LoBind tube. Discard old tube with beads.
		Proceed to the next step of the protocol.

- Combine nuclease treated libraries from previous step in groups of **8 (8 x 50 µL = 400 µL total pooled sample volume)**

- Transfer supernatant to a 1.5 mL DNA LoBind tube
- DNA concentration QC measurement is not performed after completing this 1X SMRTbell bead cleanup step

PacBio

SMRT boost bead wash of SMRTbell templates & cleanup

Perform SMRT boost bead wash followed by cleanup with 1X SMRTbell cleanup beads

1 Input DNA quality control

↓

2 Dephosphorylation

‖

3 Cas9-gRNA complex formation

↓

4 Cas-9 digestion & cleanup

↓ ○ Safe stop

5 dA tail

↓

6 Adapter ligation & cleanup

↓ ○ Safe stop

7 Nuclease Treatment

↓

8 Sample pooling & cleanup

↓ ○ Safe stop

9 SMRT boost wash & cleanup

↓

10 Concentrate samples for ABC

○ Safe stop

9.1 SMRT boost bead wash of SMRTbell templates

✔	Step	Instructions for bead washing and sample collection
	9.1	Prepare the beads by washing in SMRT boost buffer • To a 1.5 mL DNA LoBind tube, first add 1 mL of room-temperature SMRT boost buffer and then 100 µL of resuspended, room-temperature SMRT boost beads. • Pulse-vortex 5 times to mix. • Quick-spin the tube and magnetically separate for 2 minutes until the supernatant is clear. • Remove the supernatant and wash once more by adding 1 mL of SMRT boost buffer. • Pulse-vortex 5 times to mix. • Quick-spin the tube and magnetically separate for 2 minutes until the supernatant is clear. Remove the supernatant. • Resuspend the beads in 200 µL SMRT boost buffer by pulse-vortexing 5 times to mix. • Quick-spin to collect the beads.
	9.2	Prepare the SMRTbell templates for SMRT boost bead washing by mixing together one or more 8-plex pools (each at 100 µL) from Step 8. Bring the total volume to 300 µL with Elution Buffer if combining less than 3 pools of 8, as shown in the table below.

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

9.3	Add 300 µL of SMRTbell templates to 200 µL SMRT boost beads in wash buffer from step 9.1. Pulse-vortex 5 times to mix. Spin down to collect.
9.4	Gently rotate-mix for 30 minutes at RT using a rotator at low speed (~10 rpm).
9.5	Spin down to collect and magnetically separate for 2 minutes until the supernatant is clear.
9.6	Aliquot 500 µL of the SMRTbell-containing supernatant into a fresh 1.5 mL LoBind tube.

9.7 1X SMRTbell bead cleanup

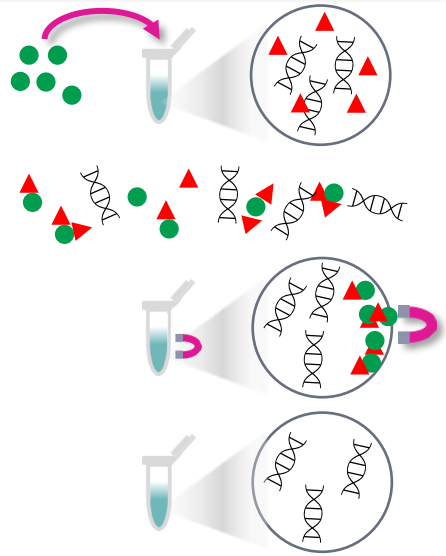
✔	Step	Instructions
	9.7	Add 500 µL of resuspended, room-temperature SMRTbell cleanup beads to 500 µL SMRTbell-containing supernatant from step 9.6.
	9.8	Pipette-mix the beads 8–10 times until evenly distributed.

- Serves to clean up & concentrate sample down from 500 µL to 100 µL

- **Note:** SMRT boost beads are **only** for use with the PureTarget repeat expansion panel SMRTbell library prep workflow – use of SMRT boost beads with other types of PacBio library construction workflows is **not supported**
- Perform one **SMRT boost** bead cleanup reaction per final pool of up to 24 PureTarget samples
- For >24 PureTarget samples → perform two SMRT boost bead cleanups with ≤24 samples each

- Prior to use, bring SMRT boost buffer and SMRT boost beads to room temperature, vortex and then spin down

- Prepare templates for SMRT boost bead washing by mixing together one or more 8-plex pools (each 100 µL)
- Bring total batch volume to **300 µL** with Elution Buffer if combining less than 3 pools of 8 (see table)



SMRT boost beads serve to prepare PureTarget repeat expansion panel libraries for sequencing and are then removed.

Concentrate samples for ABC

Perform concentration step with 1X SMRTbell cleanup beads to reduce final sample volume prior to ABC (primer annealing / polymerase binding / complex cleanup)

1 Input DNA quality control

↓

2 Dephosphorylation

‖

3 Cas9-gRNA complex formation

↓

4 Cas-9 digestion & cleanup

↓ ○ Safe stop

5 dA tail

↓

6 Adapter ligation & cleanup

↓ ○ Safe stop

7 Nuclease Treatment

↓

8 Sample pooling & cleanup

↓ ○ Safe stop

9 SMRT boost wash & cleanup

↓

10 Concentrate samples for ABC

○ Safe stop

10. Concentrating samples for ABC

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

- Add **100 µL** of SMRTbell cleanup beads to **100 µL** of sample volume containing a pooled mixture of up to 24 SMRTbell templates

Elute concentrated sample (containing up to 24 SMRTbell templates) into **16 µL** of EB

- **Take 1 µL** to perform DNA concentration QC using Qubit dsDNA HS kit
- Use remaining **15 µL** to proceed with sample setup (ABC[1])

- **QC step:** Measure DNA concentration using Qubit 1X dsDNA HS kit
- Expected recovery is **~0.02 – 0.5% (e.g., ~3 ng – 80 ng per 8-plex)** relative to starting input DNA mass
- **Note:** Significantly higher recoveries could indicate an issue with the nuclease treatment step

Proceed to **Step 11** of this Procedure & Checklist (103-329-400) to perform **ABC**[1] sample setup (**do not use** SMRT Link Sample Setup)

[1] For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) (Step 11a for Revio systems or Step 11b for Sequel II/IIe systems) – Do not use SMRT Link Sample Setup

PacBio

PureTarget sequencing preparation workflow overview

Sample setup workflow overview for PureTarget libraries – Revio system

Follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating targeted sequencing libraries without amplification*** (103-329-400) – Do not use SMRT Link Sample Setup

1 Anneal sequencing primer

↓

2 Bind sequencing polymerase

↓

3 Clean up SMRTbell complexes

↓ Safe stop

4 Internal control dilution

↓

5 Final loading dilution

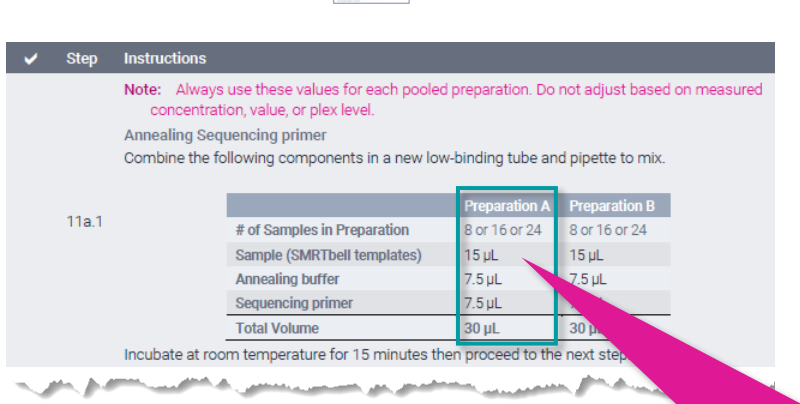
- Use entire volume (**15 µL**) of pooled SMRTbell templates per ABC reaction
- **Note:** Always use the fixed reagent volumes shown at each ABC step for each pooled batch

Revio system ABC workflow for PureTarget repeat expansion panel samples

Follow **Procedure & checklist 103-329-400** for ABC instructions[1]

8, 16 or 24 samples

ABC Preparation A
(Pool of 8/16/24 samples in 15 µL)

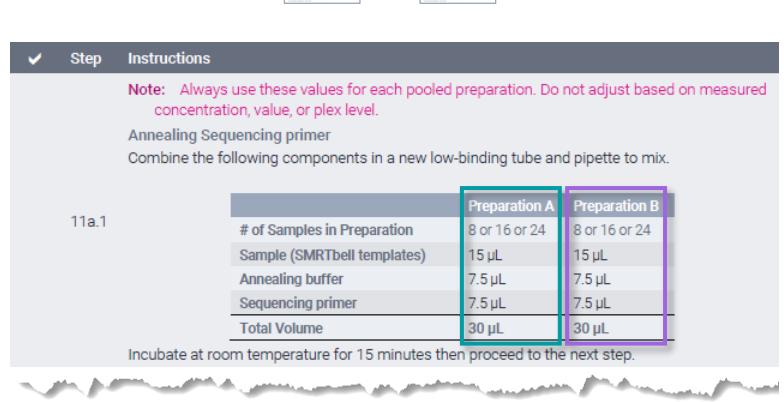


Sequence pooled samples on one Revio SMRT Cell

32, 40 or 48 samples

ABC Preparation A
(Pool of 24 samples in 15 µL)

ABC Preparation B
(Pool of 8/16/24 samples in 15 µL)



Sequence pooled samples on one Revio SMRT Cell

NOTE:
ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

[1] For primer annealing, polymerase binding & complex cleanup (ABC) steps, follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) (Step 11a for Revio systems or Step 11b for Sequel II/IIe systems) – Do not use SMRT Link Sample Setup.

Sample setup procedure for PureTarget libraries – Revio system

*A*nneal sequencing primer (*A*BC)

1. Anneal sequencing primer
2. Bind sequencing polymerase
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

Anneal sequencing primer

✔	Step	Instructions
		Note: Always use these values for each pooled preparation. Do not adjust based on measured concentration, value, or plex level.
		Annealing Sequencing primer
		Combine the following components in a new low-binding tube and pipette to mix.
	11a.1	

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

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

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

Sample setup procedure for PureTarget libraries – Revio system

*B*ind sequencing polymerase (A*B*C)

1. Anneal sequencing primer
2. **Bind sequencing polymerase**
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

Dilute sequencing polymerase

✔	Step	Instructions
		Combine the following components in a single low-bind tube and pipette to mix. The prepared volume of diluted polymerase is sufficient to process all specified samples in a batch.
	11a.2	

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

Diluted polymerase must be used immediately.

- **Note:** For PureTarget repeat expansion samples, polymerase working solution concentration after performing polymerase dilution step is **higher** than for Kinnex & WGS samples

Bind sequencing polymerase

✔	Step	Instructions
		Add annealed sample to diluted polymerase and finger tap or pipette to mix.
	11a.3	

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

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

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

Sample setup procedure for PureTarget libraries – Revio system

Clean up SMRTbell complexes (ABC)

1 Anneal sequencing primer

↓

2 Bind sequencing polymerase

↓

3 Clean up SMRTbell complexes

↓ Safe stop

4 Internal control dilution

↓

5 Final loading dilution

Purification of polymerase-bound SMRTbell complexes

✔	Step	Instructions

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

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

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

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

11a.4

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

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

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

- Equilibrate SMRTbell cleanup beads and loading buffer to room temperature at least 30 min before use

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

- **Do not** wash bead pellet with ethanol

Sample setup procedure for PureTarget libraries – Revio system

Internal control dilution

1 Anneal sequencing primer

2 Bind sequencing polymerase

3 Clean up SMRTbell complexes

Safe stop

4 Internal control dilution

5 Final loading dilution

Internal control dilution – First dilution

✔	Step	Instructions
	11a.5	Prepare only one control dilution, regardless of number of samples (up to 48 samples).
		1st Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

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

- **Prepare only <u>one</u> control dilution reaction**, regardless of number of samples (up to 48 samples per SMRT Cell for Revio system)

Internal control dilution – Second dilution

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

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

Internal control dilution – Third dilution

✔	Step	Instructions
		3rd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.
	11a.7	

Reagent	Internal Control
Dilution buffer	19 µL
Sequencing control	1.0 µL
Total Volume	20 µL

Sample setup procedure for PureTarget libraries – Revio system

Final loading dilution

1. Anneal sequencing primer
2. Bind sequencing polymerase
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

Final loading dilution

✔	Step	Instructions

Combine the following and protect from light:

11a.8

If loading ≤24 samples

# of Samples in preparation	≤24-plex
Prepared Sample (Preparation A)	48.5 µL
Loading buffer	48.5 µL
Diluted internal control (Dilution 3)	3 µL
Total volume	100 µL

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

If loading >24 samples

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

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

- If loading **≤24 samples onto Revio SMRT Cell**, add 48.5 µL Preparation A + **48.5 µL Loading buffer** + 3 µL diluted internal control
  → Transfer entire mixture (**100 µL**) to appropriate sample well in Revio sequencing plate

- If loading **>24 samples onto Revio SMRT Cell**, add 48.5 µL Preparation A + **48.5 µL Preparation B** + 3 µL diluted internal control
  → Transfer entire mixture (**100 µL**) to appropriate sample well in Revio sequencing plate

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

Sample setup workflow overview for PureTarget libraries – Sequel II/IIe system

Follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) – Do not use SMRT Link Sample Setup

1. Anneal sequencing primer
2. Bind sequencing polymerase
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

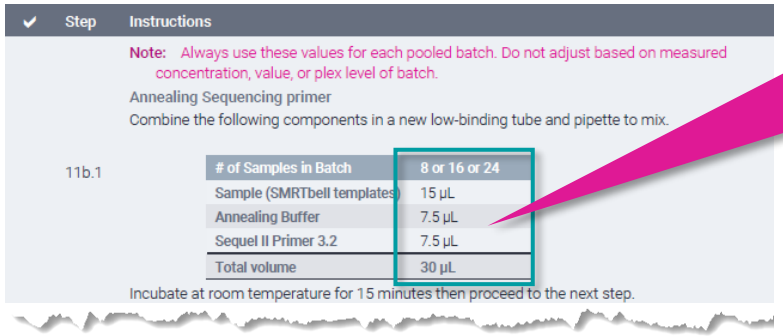
- Use entire volume (**15 µL**) of pooled SMRTbell templates per ABC reaction
- **Note:** Always use the fixed reagent volumes shown at each ABC step for each pooled batch

Sequel II/IIe system ABC workflow for PureTarget repeat expansion panel samples

8, 16 or 24 samples

Follow **Procedure & checklist 103-329-400** for ABC instructions

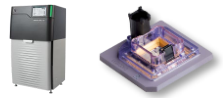
ABC[1] (Pool of 8/16/24 samples in 15 µL)



NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch



Sequence pooled samples on one Sequel II SMRT Cell

SMRT Link Run Design workflow overview for PureTarget libraries

SMRT Link run design recommendations for PureTarget repeat expansion panel samples

Select desired **application type** in SMRT Link

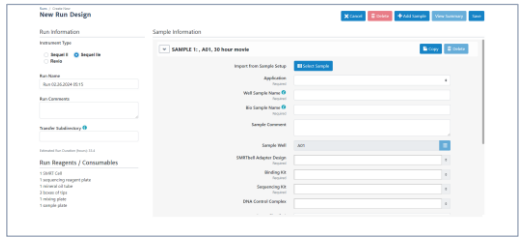
SMRT Link v13.1

Runs

Instrument Type

Sequel II Sequel IIe

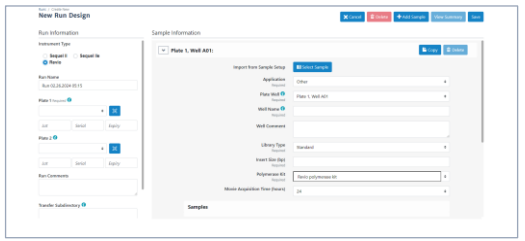
Revio



Instrument Type

Sequel II Sequel IIe

Revio



Note: *P1* loading range for PureTarget libraries generated for supported gDNA sample types can range from **5 – 35%**

Application type

PureTarget repeat expansion[1]

Whole genome sequencing
- Human WGS
- Microbial assembly
- Other WGS

RNA sequencing
- Iso-Seq method
- MAS-Seq single cell
- Kinnex single-cell RNA
- Kinnex full-length RNA

Viral sequencing
- Adeno-associated virus

Metagenomics
- Kinnex 16S rRNA
- Full-length 16S rRNA sequencing
- Shotgun metagenomic profiling or assembly

Targeted sequencing
- HiFi target enrichment
- <3kb amplicons
- >=3kb amplicons
- PureTarget repeat expansion

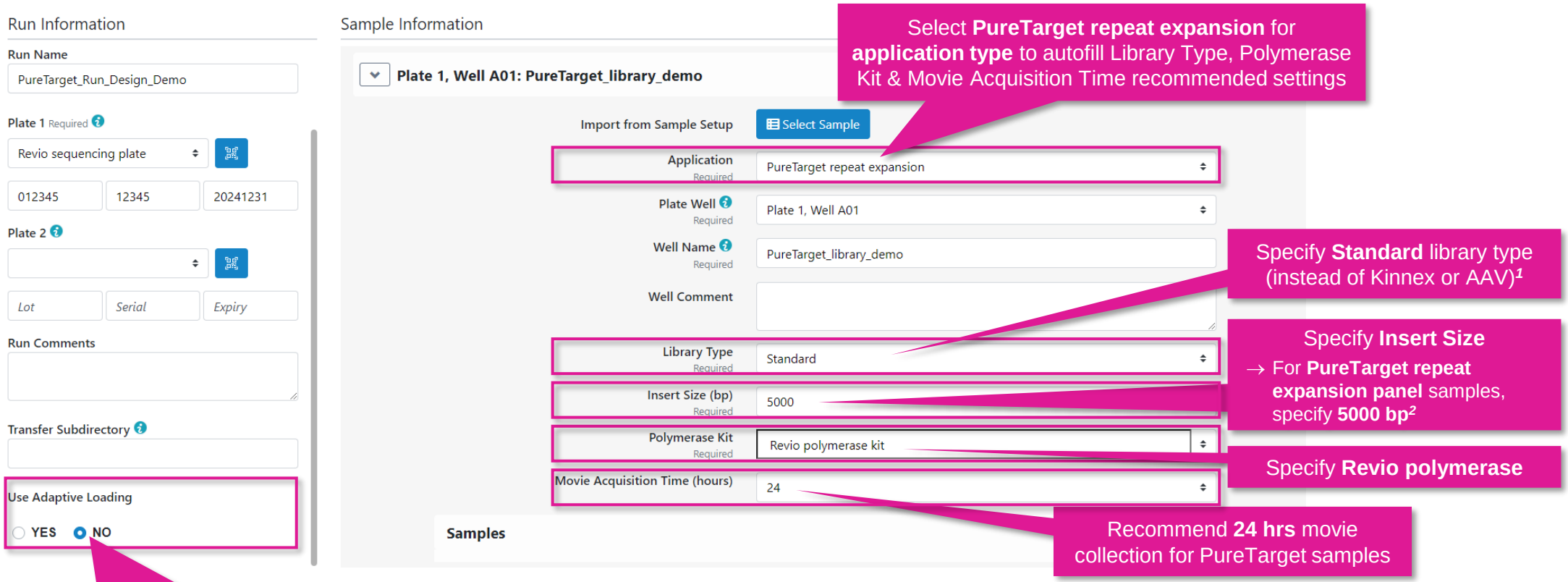
Other

SMRT Link module	Key setup parameters for PureTarget libraries	Sequel II/IIe system recommended settings for PureTarget libraries	Revio system recommended settings for PureTarget libraries
Runs → Run design	Adapter / Library type	SMRTbell Adapter Design = Overhang –SMRTbell Prep Kit 3.0	Library type = Standard
	Movie collection time	30 hrs	24 hrs
	Use adaptive loading	NO	
	On-instrument CCS	CCS Analysis Output - Include Low Quality Reads = YES CCS Analysis Output - Include Kinetics Information = YES	Consensus Mode = MOLECULE Include Base Kinetics = NO

[1] Users have two options for analysis when setting up sequencing runs in SMRT Link. For the fastest turnaround time and seamless analysis, users can include the PureTarget repeat expansion analysis in their run design and analysis will be automatically performed when sequencing is complete. Alternatively, users who prefer command line analysis may configure SMRT Link to do automatic demultiplexing only. Demultiplexed BAM files may then be transferred for command line analysis starting at the mapping step.

SMRT Link Run Design procedure for PureTarget libraries – Revio system

Run information and sample information



IMPORTANT! For Revio system PureTarget repeat expansion panel samples, specify **Use Adaptive Loading = NO**[3]

- **Note: Use Adaptive Loading field** is a run-level setting and the default value for new Revio run designs is YES – however, PureTarget repeat expansion samples require **Use Adaptive Loading = NO**
- If an attempt is made to save a Revio run design with Adaptive Loading = YES and the run includes one or more PureTarget samples, then an error window will appear prompting the user to specify **NO** for Use Adaptive Loading

[1] **Library Type** field determines which adapter finding algorithm is used during post-primary analysis.

[2] **Note:** This '5000 bp' value only serves as a **placeholder** since the **actual average insert size distribution of PureTarget repeat expansion samples may vary**.

[3] **Note:** In SMRT Link v13.1, Adaptive Loading is ON by default for all Revio system run designs. For PureTarget repeat expansional panel samples, specify **Adaptive Loading = NO** to enable correct sample immobilization conditions to be used on the Revio system. PureTarget repeat expansional panel samples should not be included in the same run design as other sample types that require Adaptive Loading to be enabled. After starting a run with PureTarget repeat expansional panel samples, users should wait until the Revio system door is unlocked to pre-load samples that require adaptive loading

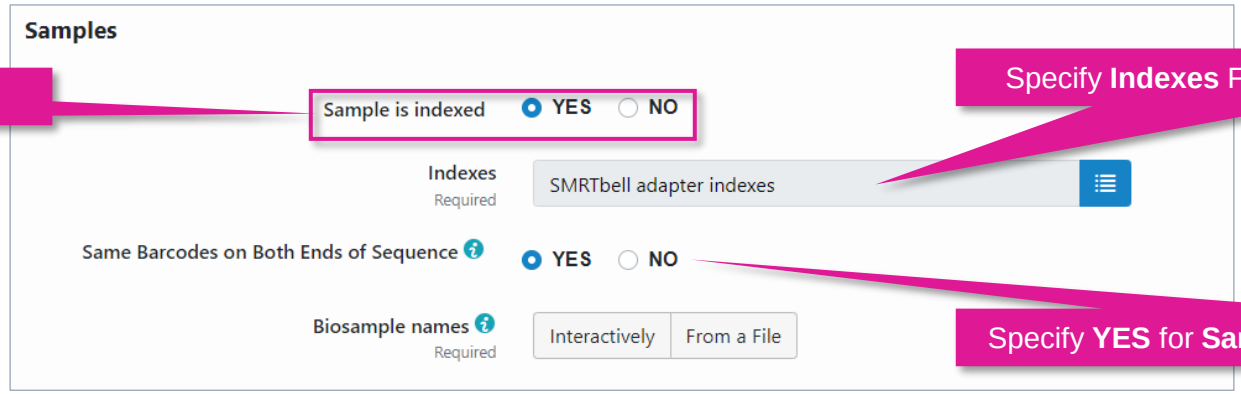
SMRT Link Run Design procedure for PureTarget libraries – Revio system (cont.)

Sample indexing (barcoding) information

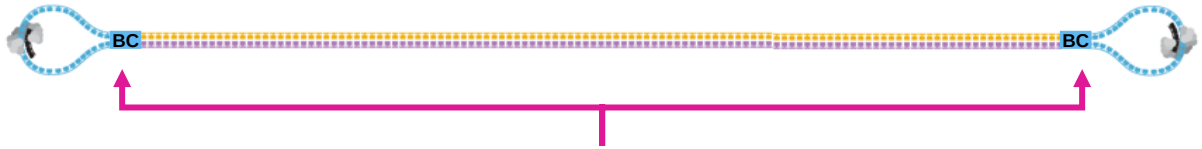
Default = **YES** for **Sample is indexed**

Specify **Indexes** FASTA = **SMRTbell adapter indexes**

Specify **YES** for **Same barcodes on both ends of sequences**



Example PureTarget library molecule containing SMRTbell indexed adapters[1] at both ends



Both forward & reverse terminal SMRTbell adapters contain the same barcode sequence

Example interactive biosample name specification for a multiplexed PureTarget library sample

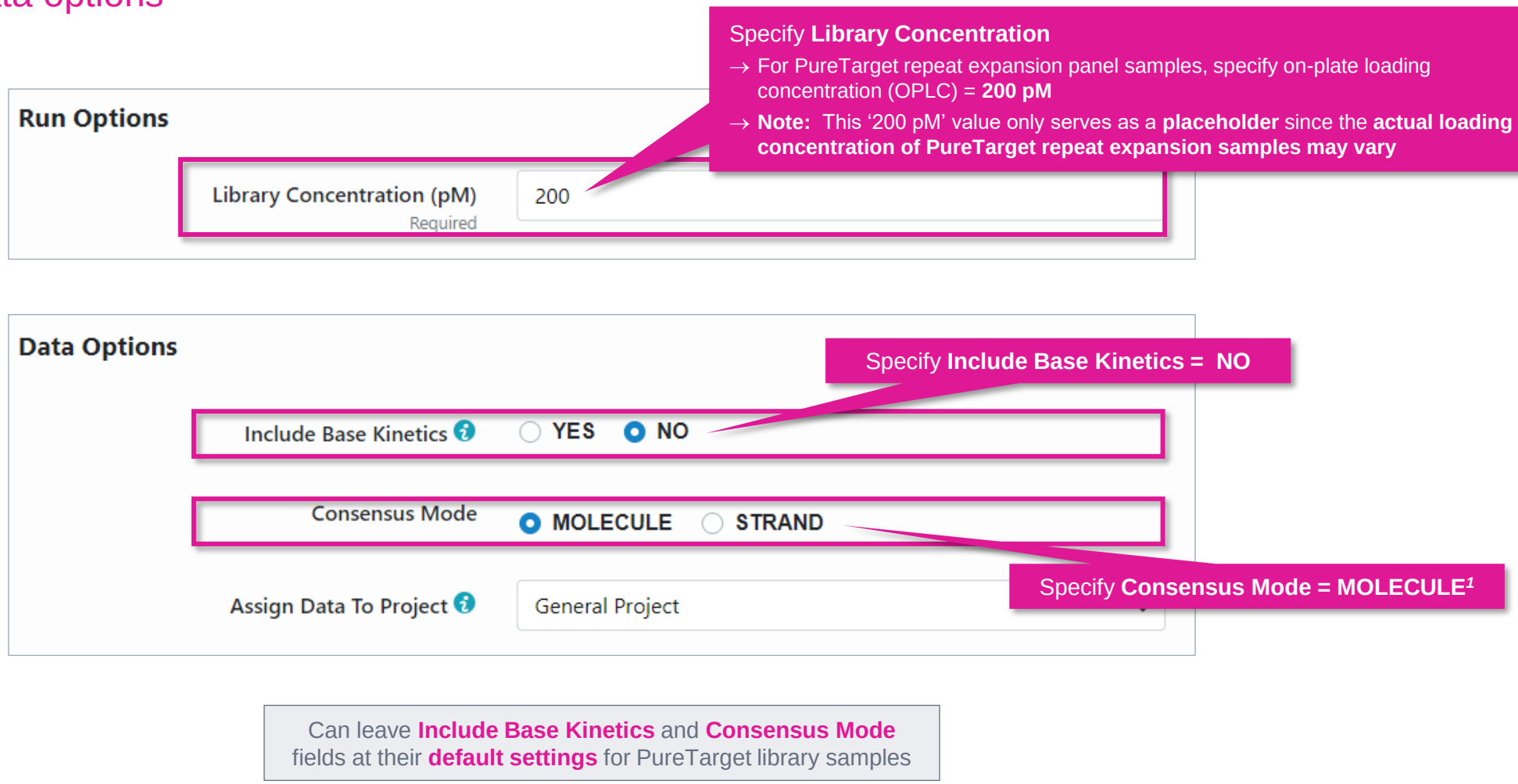
SMRTbell adapter indexes

```
>bc2001
ATCGTGCGACGAGTAT
>bc2002
TGCATGTCATGAGTAT
>bc2003
ACGAGTGCTCGAGTAT
>bc2004
TGCAGTGCTCGAGTAT
```

[1] SMRTbell adapter index plate 96A (102-009-200) contains 96 indexed SMRTbell adapters in plate format (1 sample per index). The plate includes indexes bc2001–bc2096.

SMRT Link Run Design procedure for PureTarget libraries – Revio system (cont.)

Run options and data options



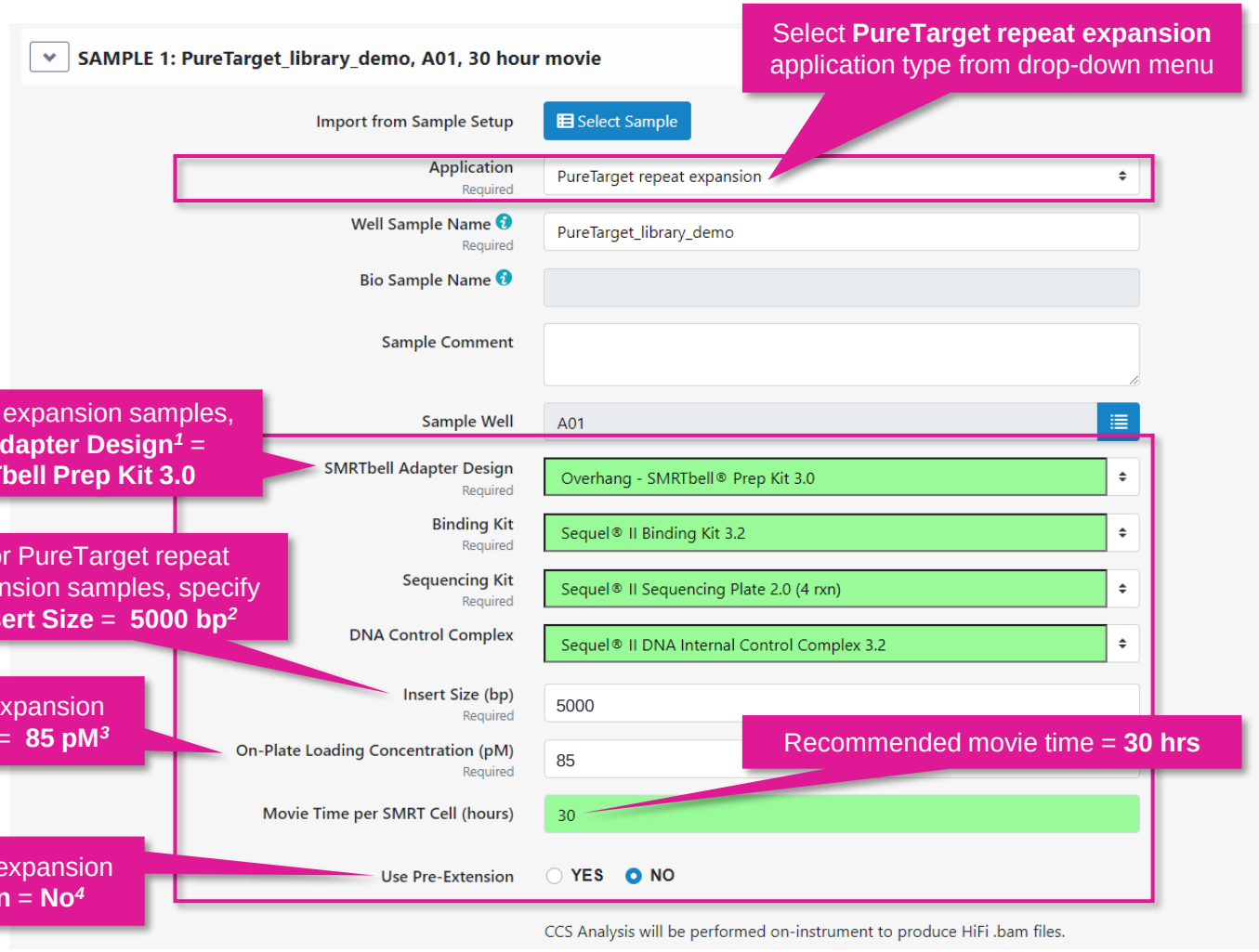
Can leave **Include Base Kinetics** and **Consensus Mode** fields at their **default settings** for PureTarget library samples

[1] **Molecule Consensus Mode** → generates a single circular consensus sequencing read for a given library insert molecule. **Strand Consensus Mode** → generates a consensus sequence for each strand (forward & reverse) of a given library insert molecule.

SMRT Link Run Design procedure for PureTarget libraries – Sequel IIe system

Sample information and run information

- Select PureTarget repeat expansion from **Application** field drop-down menu
- Following fields are auto-populated with default recommended values and high-lighted in green:
  - ❑ **SMRTbell Adapter Design**
    - → Overhang – SMRTbell Prep Kit 3.0
  - ❑ **Binding Kit**
    - → Sequel II Binding Kit 3.2
  - ❑ **Sequencing Kit**
    - → Sequel II Sequencing Plate 2.0 (4 rxn or 1 rxn)
  - ❑ **DNA Control Complex**
    - → Sequel II DNA Internal Control Complex 3.2
  - ❑ **Movie Time per SMRT Cell**
    - → 30 hrs
- For PureTarget samples, specify **Use Pre-extension = NO** (default)



Example sample information entered into a Sequel IIe system run design worksheet for a PureTarget repeat expansion panel library sample.

[1] **SMRTbell Adapter Design** field determines which adapter finding algorithm is used during post-primary analysis.

[2] **Note:** This '5000 bp' value only serves as a **placeholder** since the **actual average insert size distribution of PureTarget repeat expansion samples may vary**.

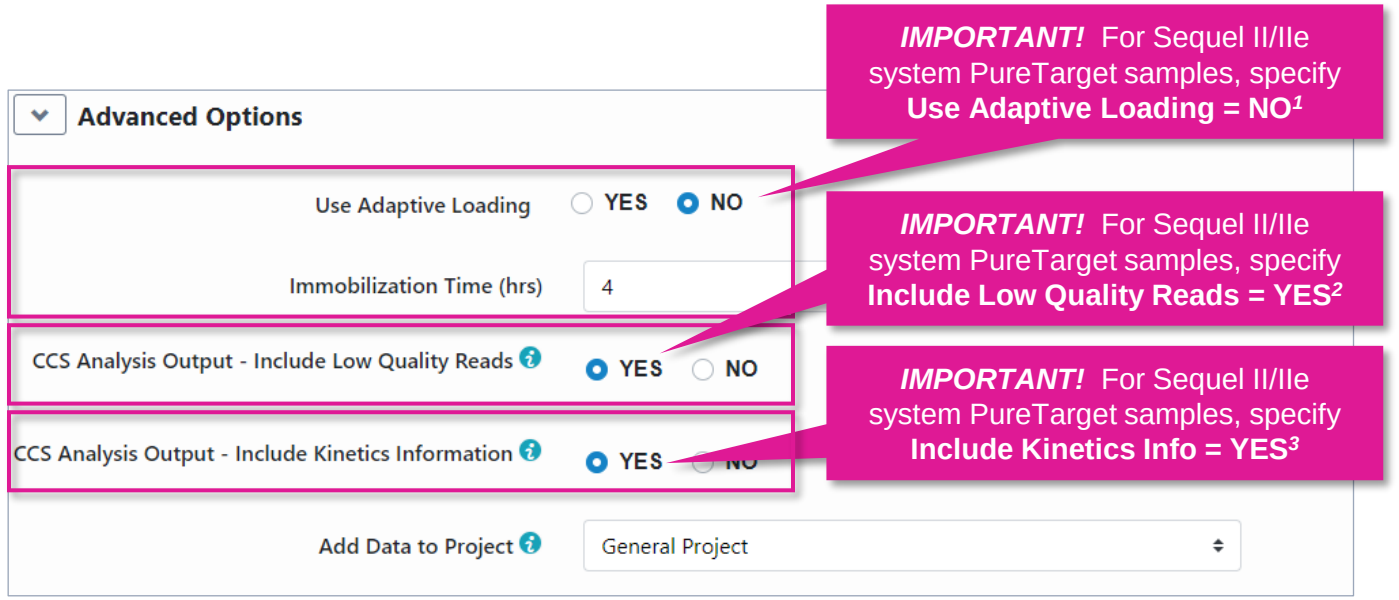
[3] **Note:** This '85 pM' value only serves as a placeholder since the actual loading concentration of PureTarget repeat expansion samples may vary.

[4] Specify **Use Pre-extension = No** to enable optimal sequencing performance for PureTarget repeat expansional panel samples.

SMRT Link Run Design procedure for PureTarget libraries – Sequel IIe system

Advanced options

- For PureTarget library samples, leave the following **Advanced Options** fields at their **default settings**
  - ❑ **Use Adaptive Loading**
    - → NO
  - ❑ **Maximum Loading Time**
    - → 4 hours
  - ❑ **CCS Analysis Output - Include Low Quality Reads**
    - → YES
  - ❑ **CCS Analysis Output - Include Kinetics Information**
    - → YES
- If desired, specify to use an alternative project folder for the **Add Data to Project** field



Example default Advanced Options settings entered into a Sequel IIe system run design worksheet for a PureTarget repeat expansion panel library sample.

[1] Post-Cas9 digestion & cleanup yields typically range from ~50% to ~100% (1 – 2 µg) per sample when using supported genomic DNA types for PureTarget library construction.

[2] For PureTarget repeat expansion panel samples, low quality reads are saved by default to enable improved sensitivity for genotyping expanded tandem repeat alleles.

[3] Since low quality reads are saved by default for PureTarget libraries, 5mC detection cannot be performed on-instrument for Sequel IIe systems and, therefore, kinetics information must be saved in the CCS analysis output file to enable base modification detection analysis to be performed in SMRT Link.

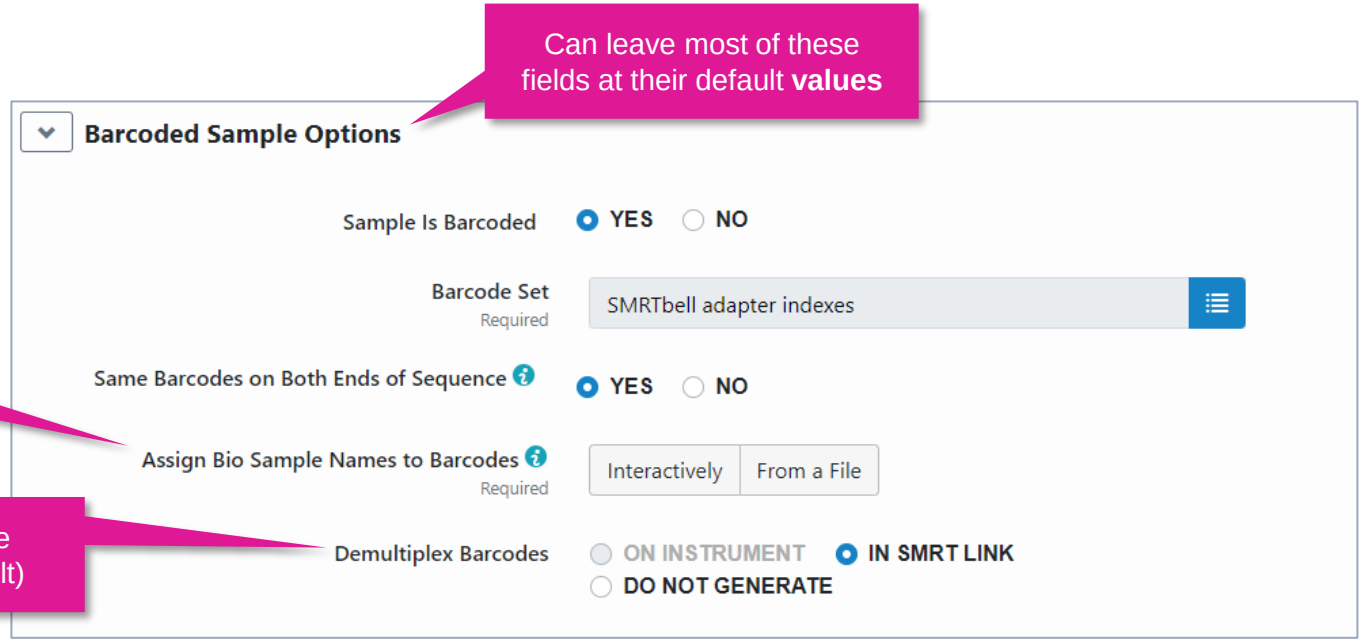
SMRT Link Run Design procedure for PureTarget libraries – Sequel IIe system

Barcoded sample options

- For PureTarget library samples, can leave most **Barcoded Sample Options** fields at their **default settings**

Specify Bio Sample Names, either interactively or by downloading a CSV file (**Interactively** or **From a file**)

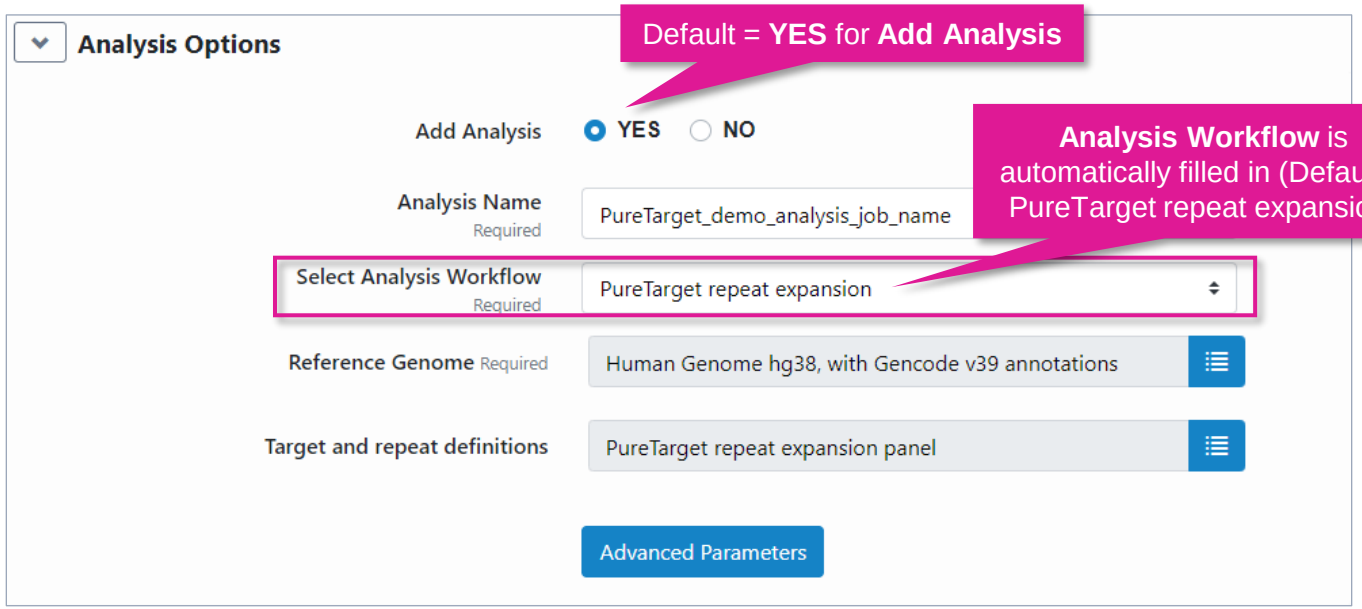
Optionally specify to perform barcode demultiplexing **in SMRT Link**[1] (Default)



Example default Barcoded Sample Options settings entered into a Sequel IIe system run design worksheet for a PureTarget repeat expansion panel library sample.

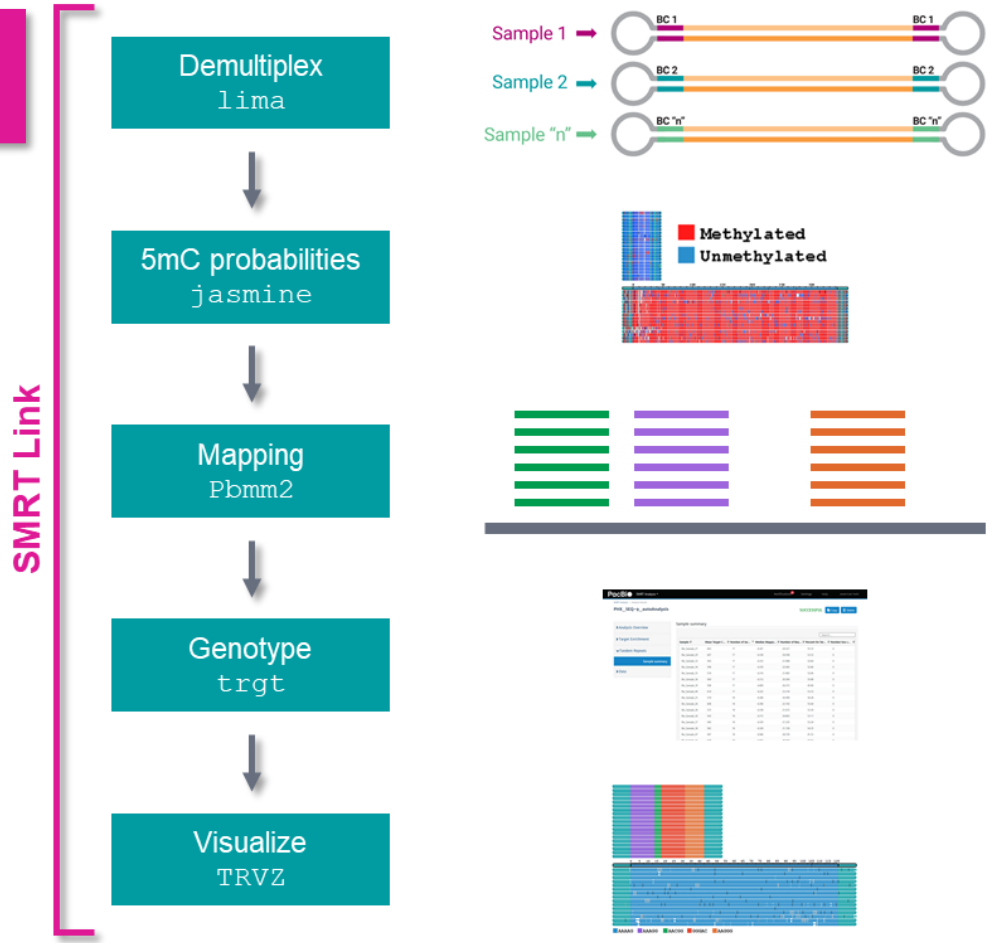
[1] Since low quality reads are saved by default for PureTarget libraries, barcode demultiplexing cannot be performed on-instrument and **must be performed in SMRT Link**.

SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems



- Analyze multiplexed PureTarget panel samples using PureTarget repeat expansion analysis application[1]
- The analysis produces target enrichment summary statistics and uses the tandem repeat genotyping tool (TRGT) for variant calling and visualization

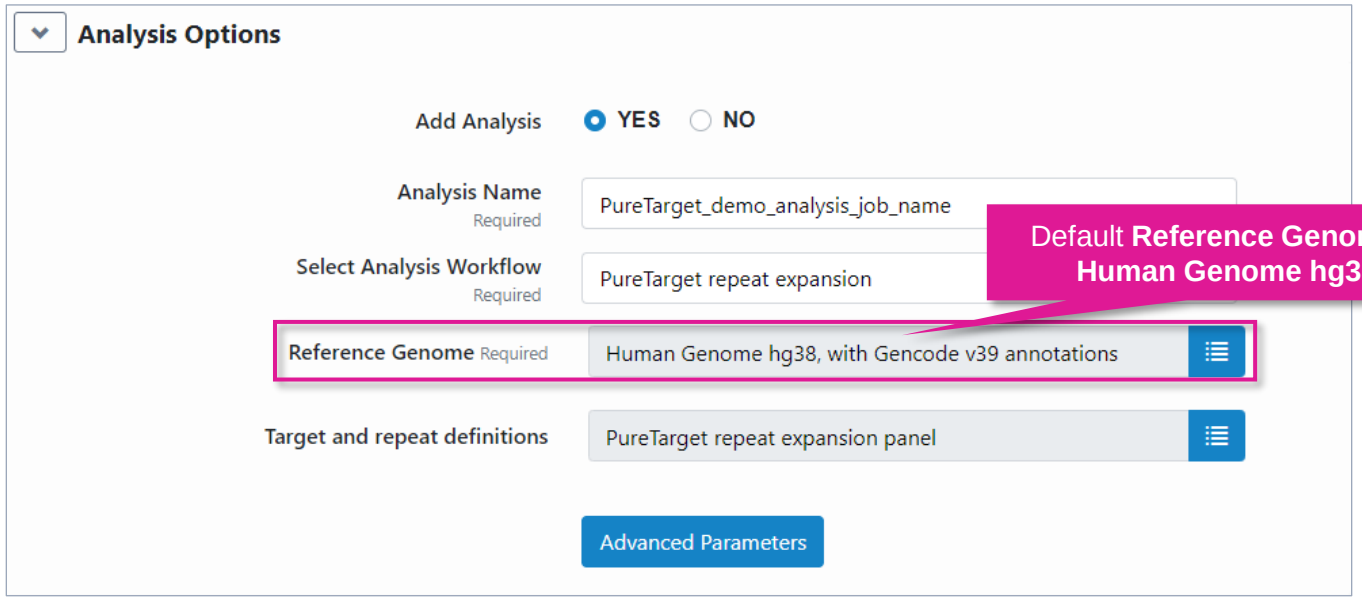
PureTarget repeat expansion analysis application



[1] Users have two options for analysis when setting up sequencing runs in SMRT Link. For the fastest turnaround time and seamless analysis, users can include the PureTarget repeat expansion analysis in their run design and analysis will be automatically performed when sequencing is complete. Alternatively, users who prefer command line analysis may configure SMRT Link to do automatic demultiplexing only. Demultiplexed BAM files may then be transferred for command line analysis starting at the mapping step..

[2] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for PureTarget repeat expansion analysis application.

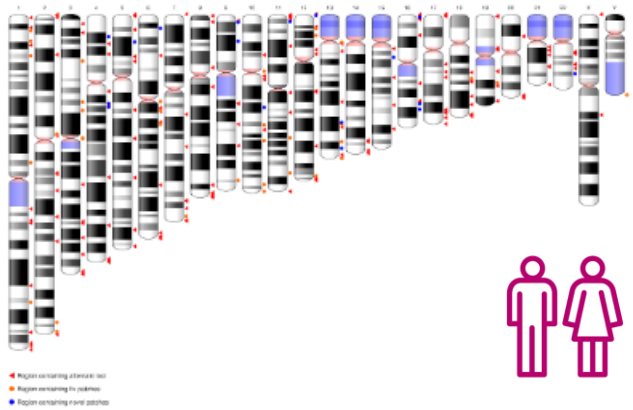
SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems



Default **Reference Genome = Human Genome hg38**

- Specify a reference genome against which to align the reads
- Default set = Human Genome hg38, with Gencode v39 annotations

Reference genome specification

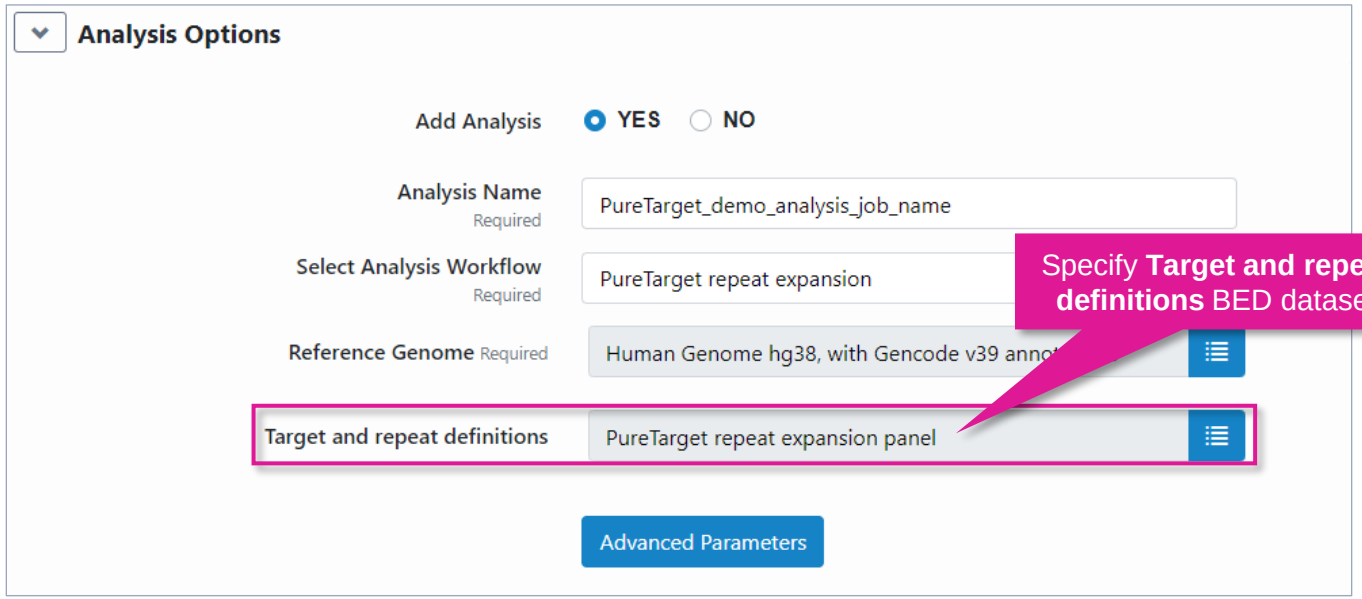


pbmm2

Sequence alignment program for aligning PacBio sequencing data against a reference database

[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for PureTarget repeat expansion analysis application.

SMRT Link Run Design analysis options for Revio system and Sequel II/IIe systems



- Specify a target and repeat definition (browser extensible data) BED dataset
  - The default set is PureTarget repeat expansion panel
- Only reads that map within the target regions in the BED file are included in the analysis
  - To “in-silico” mask data from targets included in the 20 gene Repeat Expansion panel, create a new BED dataset **without** that target
- To analyze data from a custom panel, create a new BED dataset with those additional targets
  - Note that this analysis workflow will **only** work for tandem repeat regions
  - See TRGT documentation in GitHub for more information on the required BED file format for tandem repeats

Target and repeat definitions specification

`*.PureTarget_repeat_expansion_panel.bedset.xml`

Example tandem repeat definition

```
chr4  3074876  3074966  ID=HTT,MOTIFS=CAG,CCG;STRUC=(CAG)nCAACAG(CCG)n
```

- Repeat region has coordinates chr4:3074876-3074966
- Identifier is HTT
- Region contains two tandem repeats with motifs CAG and CCG and these tandem repeats are expected to be separated by a short interrupting sequence CAACAG

TRGT

Tandem repeat genotyping tool for PacBio sequencing data

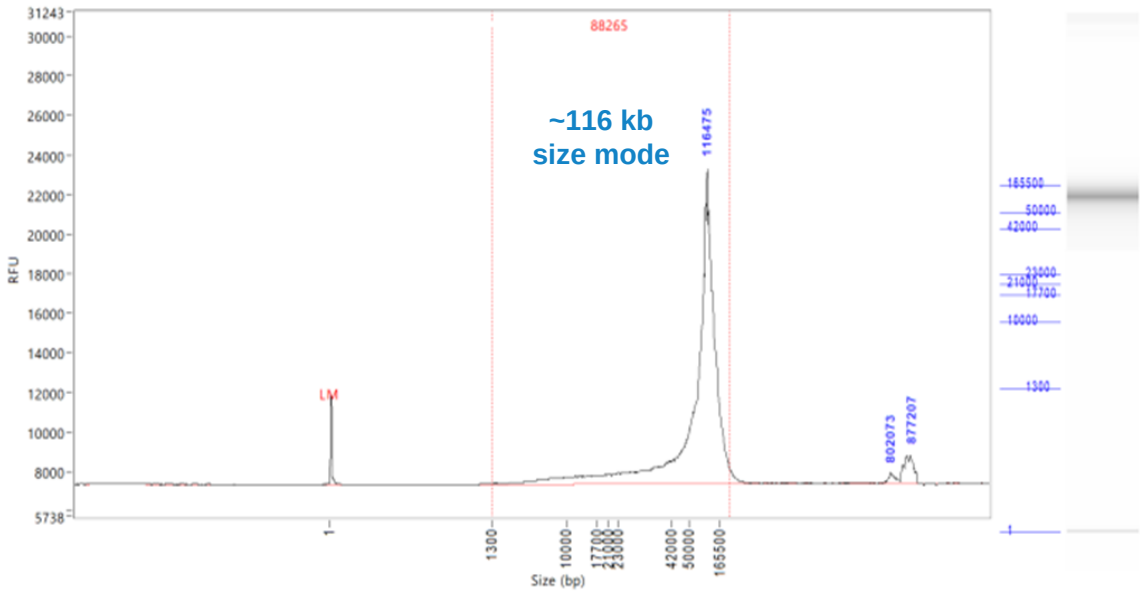
[1] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for PureTarget repeat expansion analysis application.

PureTarget example sequencing performance data

Example PureTarget repeat expansion panel library preparation QC results

Multiplexed PureTarget library prepared with genomic DNA isolated from human blood samples

Input genomic DNA sizing QC



Example Femto Pulse genomic DNA sizing QC analysis results for high-molecular weight genomic DNA extracted from a human whole blood sample using Nanobind PanDNA kit.

PureTarget library preparation step yields

Library preparation step	Yield
Starting input genomic DNA	2,000 ng per sample
Post-Cas9 digestion & cleanup yield[1]	1,340 ng (67%) per sample
Post-Adapter ligation & cleanup yield[2]	960 ng (48%) per sample
Post-nuclease treatment & final library cleanup yield[3]	10 ng (0.063%) per 8-plex

Example library preparation step yield results for PureTarget repeat expansion library prepared from a human whole blood sample.

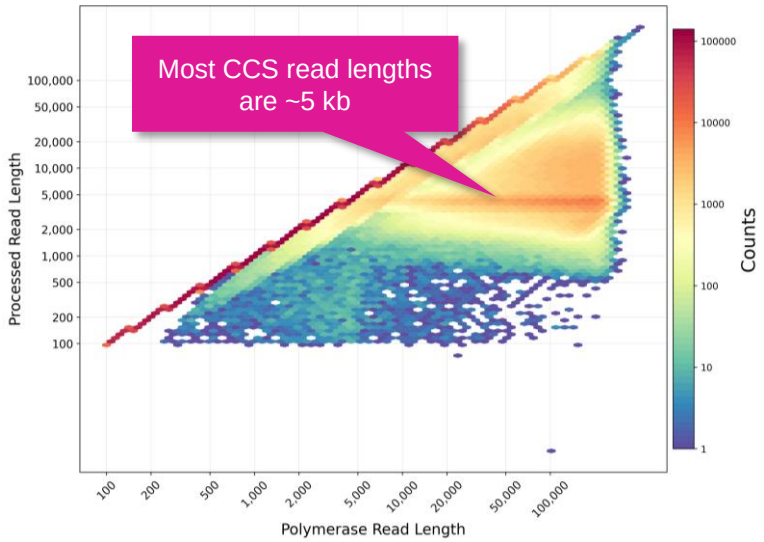
Final PureTarget library yield is typically sufficient to load **1 SMRT Cell**

[1] Post-Cas9 digestion & cleanup yields typically range from ~50% to ~100% (1 – 2 μg) per sample when using supported genomic DNA types for PureTarget library construction.
[2] Post-Adapter ligation & cleanup yields typically range from ~50% to ~100% (1 – 2 μg) per sample when using supported genomic DNA types for PureTarget library construction.
[3] Post-nuclease treatment & final cleanup yields typically range from ~0.02% to ~0.5% (3 – 80 ng) per 8-plex when using supported genomic DNA types for PureTarget library construction.

Example sequencing performance for 48-plex PureTarget repeat expansion panel library prepared from human blood samples (Revio system)

48-plex PureTarget repeat expansion panel Revio system example data[1]

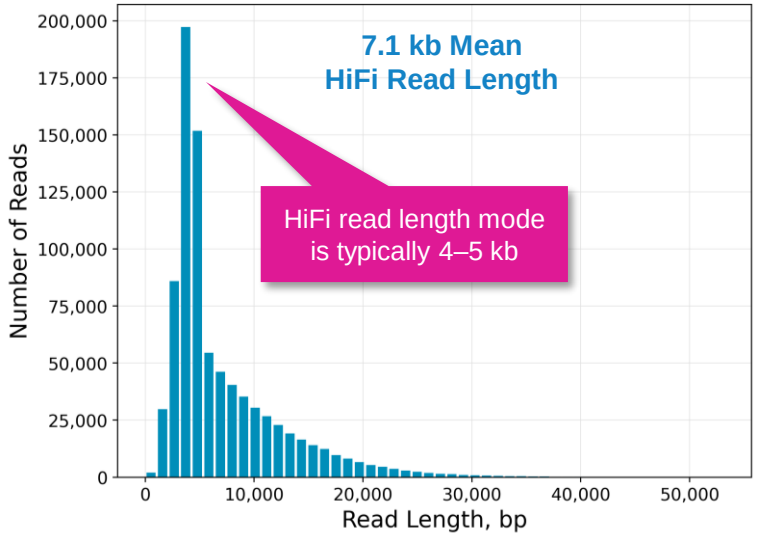
Raw Data Report



Raw Base Yield	161 Gb
Mean Polymerase Read Length	18.0 kb
P0	64%
P1	36%
P2	1%

Example metrics for a 48-plex PureTarget repeat expansion panel library run on a Revio system with Revio polymerase kit using a 24-hrs movie time. Revio system *P1* range for 48-plex PureTarget repeat expansional panel libraries was typically ~25%–38%.

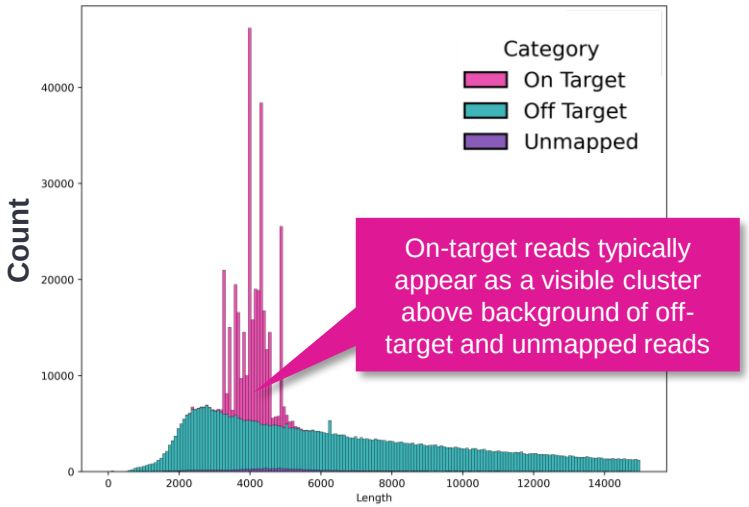
HiFi Read Length



HiFi Reads	838.2 K
HiFi Base Yield	6.0 Gb
Mean HiFi Read Length	7.1 kb
Median HiFi Read Quality	Q38
HiFi Read Mean # of Passes	21

For 48-plex PureTarget repeat expansion panel libraries, per-Revio SMRT Cell HiFi read counts were typically ~0.8 M–1.0 M depending on the final library insert size and *P1* loading performance.

Target Enrichment Summary Metrics



Total Bases*	6,547,987,725
Total Reads*	886,509
Median Read Length*	4,855 bp
Median Read Quality*	Q36
Sample Count	48
Target Regions	20

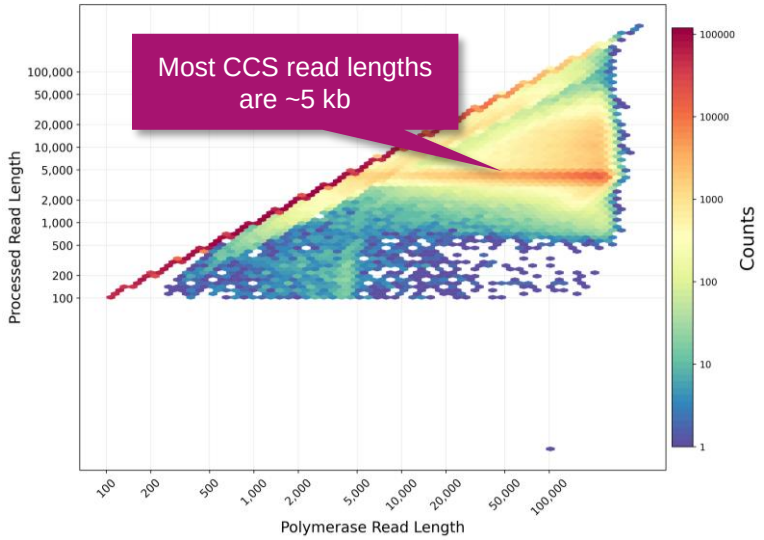
*** Includes HiFi + non-HiFi data.** For 48-plex PureTarget repeat expansion panel libraries, per-Revio SMRT Cell total read counts were typically ~0.8 M–1.0 M.

[1] Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in lower data yields per Revio SMRT Cell.

Example sequencing performance for 24-plex PureTarget repeat expansion panel library prepared from human blood samples (Revio system)

24-plex PureTarget repeat expansion panel Revio system example data[1]

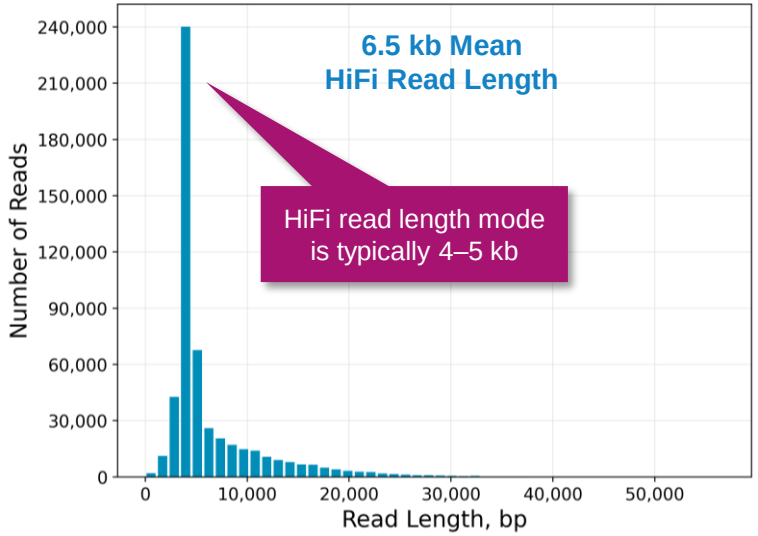
Raw Data Report



Raw Base Yield	98 Gb
Mean Polymerase Read Length	16.1 kb
P0	75%
P1	24%
P2	0%

Example metrics for a 24-plex PureTarget repeat expansion panel library run on a Revio system with Revio polymerase kit using a 24-hrs movie time. Revio system *P1* range for 24-plex PureTarget repeat expansional panel libraries was typically ~15%–30%.

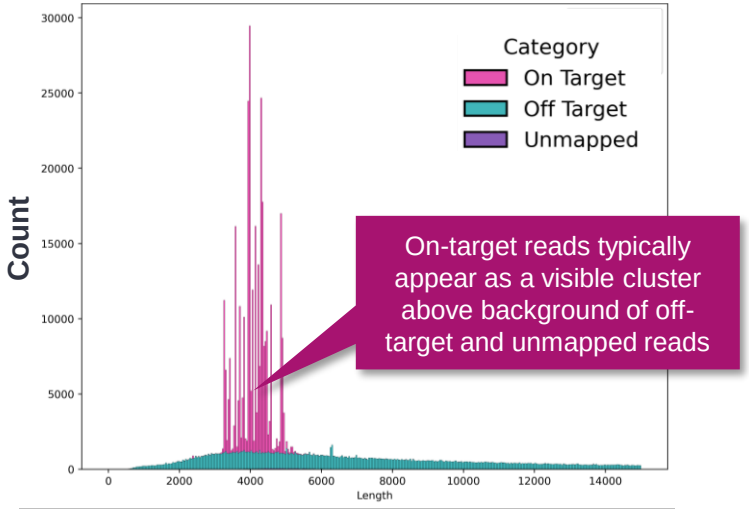
HiFi Read Length



HiFi Reads	522.6 K
HiFi Base Yield	3.37 Gb
Mean HiFi Read Length	6.5 kb
Median HiFi Read Quality	Q43
HiFi Read Mean # of Passes	25

For 24-plex PureTarget repeat expansion panel libraries, per-Revio SMRT Cell HiFi read counts were typically ~0.3 M–0.5 M depending on the final library insert size and *P1* loading performance.

Target Enrichment Summary Metrics



Total Bases*	3,648,915,399
Total Reads*	553,106
Median Read Length*	4,342 bp
Median Read Quality*	Q41
Sample Count	24
Target Regions	20

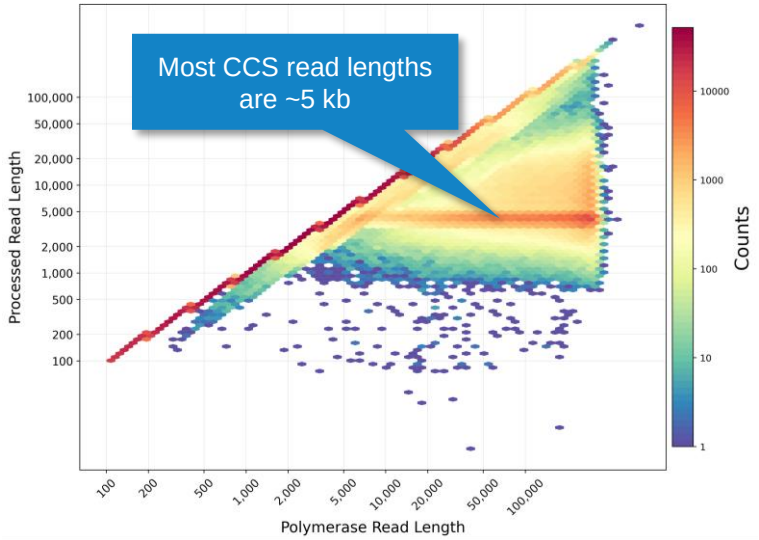
*** Includes HiFi + non-HiFi data.** For 24-plex PureTarget repeat expansion panel libraries, per-Revio SMRT Cell total read counts were typically ~0.3 M–0.5 M.

[1] Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in lower data yields per Revio SMRT Cell.

Example sequencing performance for 24-plex PureTarget repeat expansion panel library prepared from human blood samples (Sequel IIe system)

24-plex PureTarget repeat expansion panel Sequel IIe system example data[1]

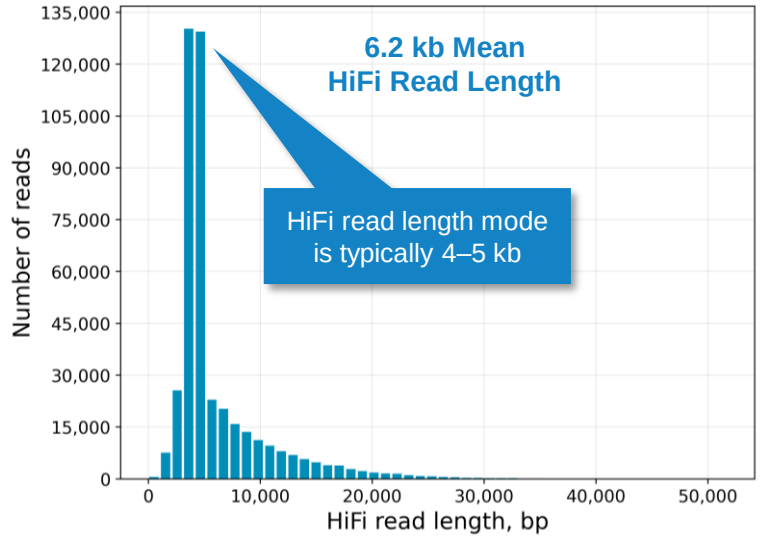
Raw Data Report



Raw Base Yield	96 Gb
Mean Polymerase Read Length	29.0 kb
P0	56%
P1	41%
P2	2%

Example metrics for a 24-plex PureTarget repeat expansion panel library run on a Sequel IIe system with Sequel II binding kit 3.2 using a 30-hrs movie time. Sequel IIe system *P1* range for 24-plex PureTarget repeat expansional panel libraries was typically ~5%-50%.

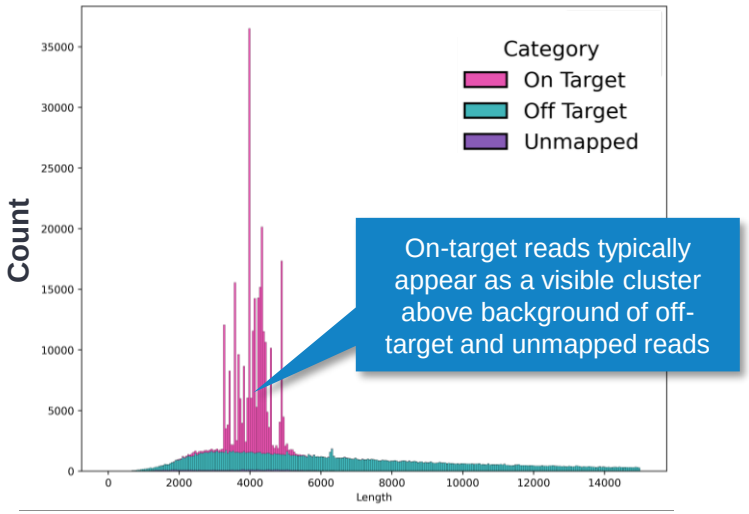
HiFi Read Length



HiFi Reads	435.8 K
HiFi Base Yield	2.7 Gb
Mean HiFi Read Length	6.2 kb
Median HiFi Read Quality	Q40
HiFi Read Mean # of Passes	18

For 24-plex PureTarget repeat expansion panel libraries, per-Sequel II SMRT Cell HiFi read counts were typically ~0.4 M–0.5 M depending on the final library insert size and *P1* loading performance.

Target Enrichment Summary Metrics



Total Bases*	3,679,524,534
Total Reads*	522,722
Median Read Length*	4,374 bp
Median Read Quality*	Q37
Sample Count	24
Target Regions	20

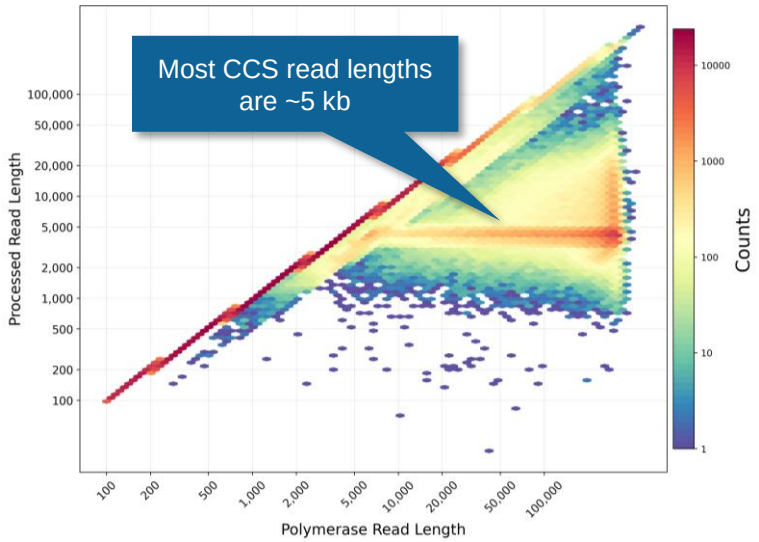
*** Includes HiFi + non-HiFi data.** For 24-plex PureTarget repeat expansion panel libraries, per-Sequel II SMRT Cell total read counts were typically ~0.4 M–0.5 M.

[1] Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in lower data yields per Sequel II SMRT Cell.

Example sequencing performance for 8-plex PureTarget repeat expansion panel library prepared from human blood samples (Sequel IIe system)

8-plex PureTarget repeat expansion panel Sequel IIe system example data[1]

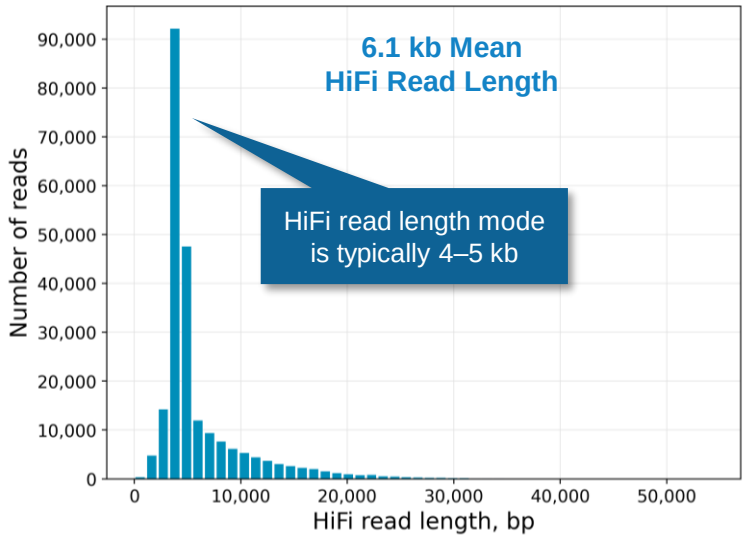
Raw Data Report



Raw Base Yield	53 Gb
Mean Polymerase Read Length	31.3 kb
P0	78%
P1	21%
P2	1%

Example metrics for a 8-plex PureTarget repeat expansion panel library run on a Sequel IIe system with Sequel II binding kit 3.2 using a 30-hrs movie time. Sequel IIe system *P1* range for 8-plex PureTarget repeat expansional panel libraries was typically ~5%-35%.

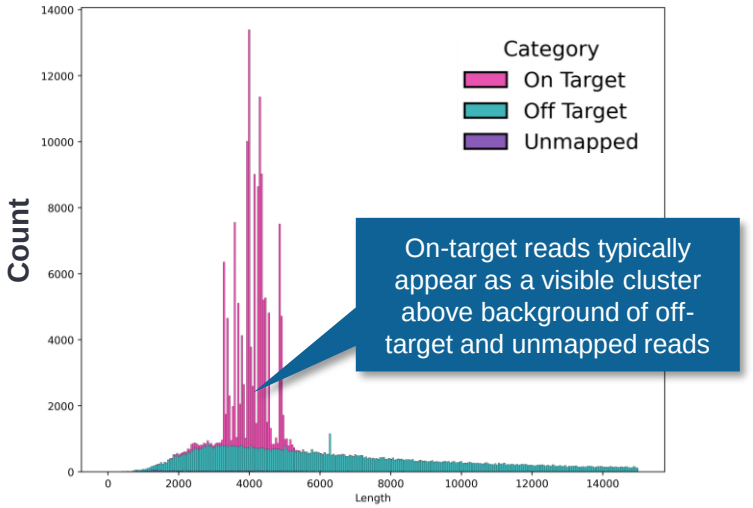
HiFi Read Length



HiFi Reads	225.4 K
HiFi Base Yield	1.4 Gb
Mean HiFi Read Length	6.1 kb
Median HiFi Read Quality	Q43
HiFi Read Mean # of Passes	20

For 24-plex PureTarget repeat expansion panel libraries, per-Sequel II SMRT Cell HiFi read counts were typically ~0.1 M–0.2 M depending on the final library insert size and *P1* loading performance.

Target Enrichment Summary Metrics



Total Bases*	1,671,935,304
Total Reads*	255,625
Median Read Length*	4,325 bp
Median Read Quality*	Q41
Sample Count	8
Target Regions	20

*** Includes HiFi + non-HiFi data.** For 24-plex PureTarget repeat expansion panel libraries, per-Sequel II SMRT Cell total read counts were typically ~0.1 M–0.2 M.

[1] Read lengths, reads/data per SMRT Cell and other sequencing performance results can vary depending on DNA sample quality, insert size, *P1* loading performance & movie time. Note: Shorter library insert sizes (<5 kb), lower DNA quality samples, and suboptimal *P1* loading performance may result in lower data yields per Sequel II SMRT Cell.

Example coverage performance for PureTarget repeat expansional panel library (Revio system)

On-target coverage results for one representative human blood sample from a 24-plex PureTarget library

DNA sample preparation

- 2 µg DNA per sample extracted from 24 human whole blood samples
- *Procedure & checklist – Extracting HMW DNA from human whole blood with RBC lysis using the Nanobind PanDNA kit* (103-377-500)

PureTarget library preparation

- 20-gene target panel library constructed using PureTarget repeat expansional panel kit (103-390-400)
- Multiplexed PureTarget library containing 24 human DNA samples

Sequencing run design

- Revio system
- 24 hrs movie time
- No adaptive loading

Coverage (per gene per sample)

- Mean = 387
- Max = 678
- Min = 74

Gene	Motif	Motif repeat number and PureTarget sequencing coverage			
		Allele 1	Coverage	Allele 2	Coverage
ATN1	CAG	19	207	17	236
ATXN2	GCT	22	240	23	234
ATXN8	CTA	9	271	11	254
ATXN3	GCT	16	204	20	232
CACNA1A	CTG	13	185	13	184
ATXN10	ATTCT	13	251	13	242
ATXN7	GCA	10	194	12	199
PPP2R2B	GCT	10	217	12	238
ATXN1	TGC	31	174	35	170
TBP	GCA	34	199	37	206
FXN	GAA	8	123	9	137
DMPK	CAG	13	163	19	203
C9ORF72	GGCCCC	5	184	5	165
TCF4	CAG	17	241	25	232
PABPN1	GCG	6	205	6	222
AR	GCA	21	421		
RFC1	AAAAG	10	94	10	94
CNBP	CAGG	15	258	15	257
FMR1	CGG	29	304	31	278
HTT	CAG	16	155	21	138

Example coverage performance for PureTarget repeat expansional panel library (Revio system) (cont.)

On-target coverage results for selected repeat expansions in different human cell line samples

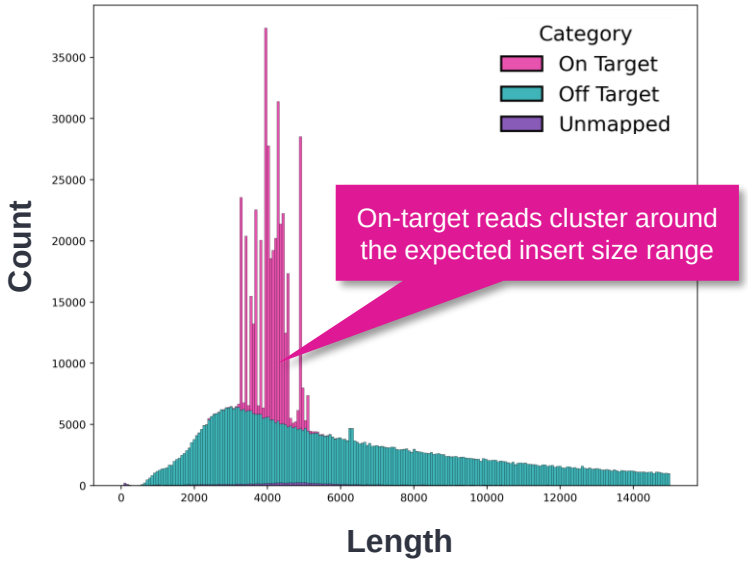
Gene		Motif	Motif repeat number and PureTarget sequencing coverage			
			Allele 1	X Coverage	Allele 2	X Coverage
FXN	NA14519	GAA	9	324	1048	243
FMR1	NA07537	CGG	29	462	336	475
FMR1	NA06968	CGG	33	157	113	56
FXN	NA16212	GAA	8	54	509	35
PABPN1	NA23629	GCG	6	363	9	397
AR	NA23709	GCA	48	194		
C9orf72	ND06751	GGCCCC	8	166	731	69

PureTarget repeat expansional panel kit can accurately detect expanded alleles containing >100 repeat expansions

Recommended guidance for evaluating PureTarget repeat expansion panel sequencing run performance

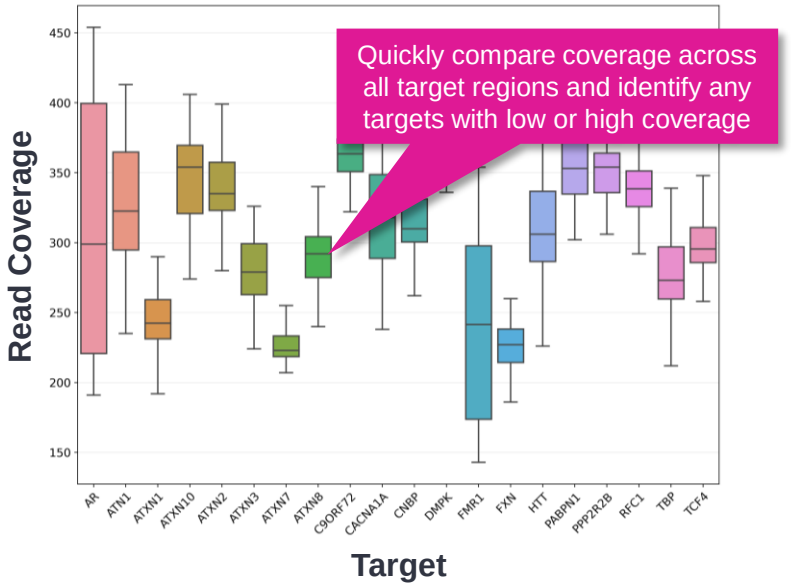
When evaluating PureTarget runs, it is generally more useful to examine the **secondary analysis results** (e.g., on-target coverage) since primary sequencing metrics like Productivity (*P0, P1, P2*) are mostly dominated by 'background' non-targeted reads

Read Categories[1]



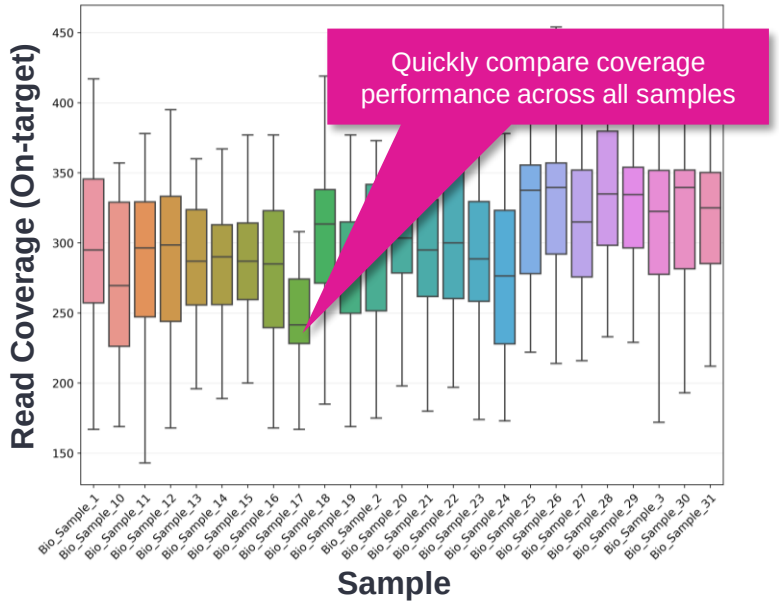
- Histogram mapping the length of On target, Off target, and Unmapped reads in the sample

Target Coverage



- Box plot for each target regions of mean coverage across all samples analyzed

Sample Coverage

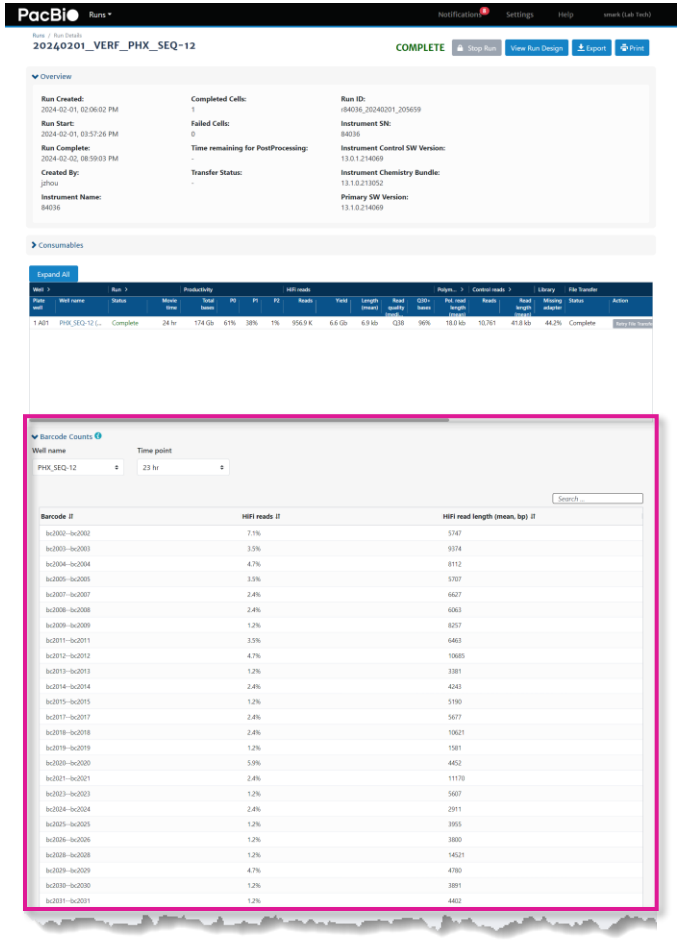


- Box plot for each sample of mean coverage across all target regions

[1] **Note:** A read is defined as being on-target if its alignment region in the reference genome has a non-empty overlap with any defined target in the input BED file.

Recommended guidance for evaluating PureTarget repeat expansion panel sequencing run performance (cont.)

For Revio system, Barcode Counts preview metrics[1] in SMRT Link Run Details report are useful for early evaluation of PureTarget sample demultiplexing performance and per-sample mean HiFi read length



Barcode Counts

Well name: PHX_SEQ-12 | Time point: 23 hr

HiFi read length, mean: An estimate of the average HiFi read length for each barcode or for unbarcoded reads

Barcode: An individual barcode detected in the sample, as well as unbarcoded reads

HiFi reads: An estimate of the % of reads with each barcode, as well as the % of unbarcoded reads

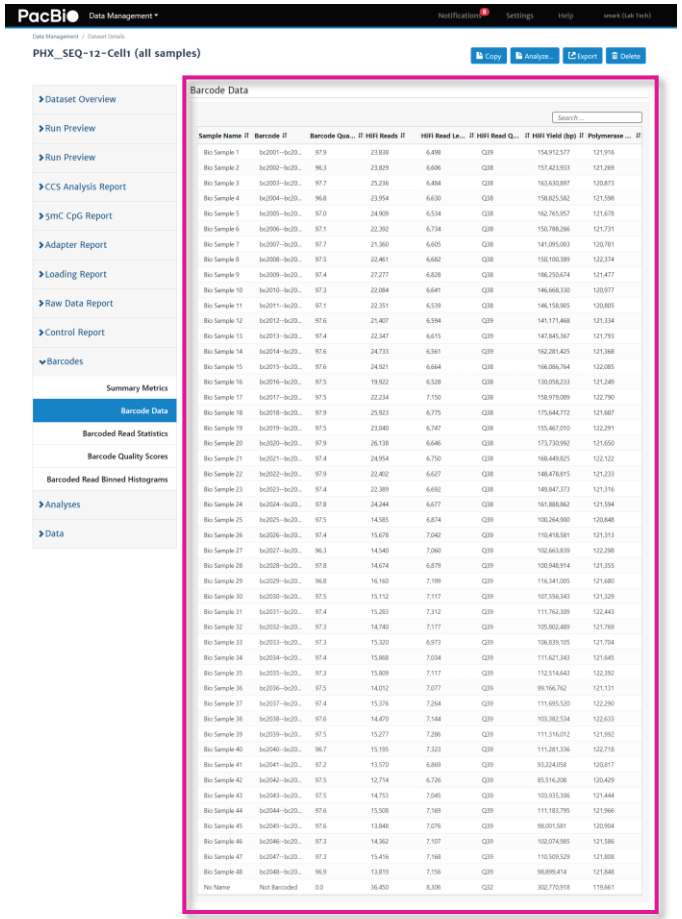
Search ...

Barcode	HiFi reads	HiFi read length (mean, bp)
bc2002--bc2002	7.1%	5747
bc2003--bc2003	3.5%	9374
bc2004--bc2004	4.7%	8112
bc2005--bc2005	3.5%	5707
bc2007--bc2007	2.4%	6627
bc2008--bc2008	2.4%	6063
bc2009--bc2009	1.2%	8257
bc2011--bc2011	3.5%	6463
bc2012--bc2012	4.7%	10685
bc2013--bc2013	1.2%	3381
bc2014--bc2014	2.4%	4243
bc2047--bc2047	3.5%	4884
bc2048--bc2048	1.2%	4294
Not Barcoded	5.9%	6508

[1] **Note:** The Barcode Counts preview values displayed may overestimate the number of unbarcoded reads. In addition, all estimates may be less accurate for barcodes at low frequency (<10%) due to sample size. Any barcodes below a 1% frequency are not displayed, and are grouped into the "Other" category

Recommended guidance for evaluating PureTarget repeat expansion panel sequencing run performance (cont.)

View Barcode demultiplexing results in SMRT Link SMRT Analysis to perform more detailed evaluation of PureTarget sample demultiplexing performance



PacBio Data Management ▾ Notifications 8 Settings Help smark (Lab Tech)

Data Management / Dataset Details

PHX_SEQ-12-Cell1 (all samples)

Copy | Analyze... | Export | Delete

- Dataset Overview
- Run Preview
- Run Preview
- CCS Analysis Report
- 5mC CpG Report
- Adapter Report
- Loading Report
- Raw Data Report
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  - Barcoded Read Statistics
  - Barcode Quality Scores
  - Barcoded Read Binned Histograms
- Analyses
- Data

Barcode Data

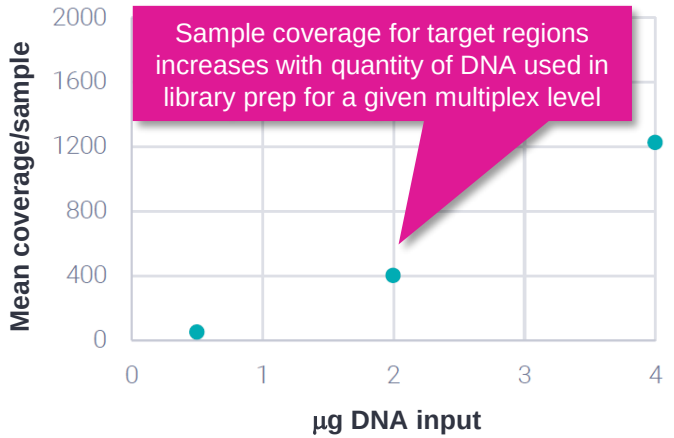
Search ...

Sample Name	Barcode	Barcode Qua...	HiFi Reads	HiFi Read Le...	HiFi Read Q...	HiFi Yield (bp)	Polymerase ...
Bio Sample 1	bc2001--bc20...	97.9	23,838	6,498	Q39	154,912,577	121,916
Bio Sample 2	bc2002--bc20...	96.3	23,829	6,606	Q38	157,423,933	121,269
Bio Sample 14	bc2014--bc20...	97.6	24,733	6,561	Q39	162,281,425	121,368
Bio Sample 15	bc2015--bc20...	97.6	24,921	6,664	Q38	166,086,764	122,085
Bio Sample 16	bc2016--bc20...	97.5	19,922	6,528	Q38	130,058,233	121,249
Bio Sample 17	bc2017--bc20...	97.5	22,234	7,150	Q38	158,979,009	122,790
Bio Sample 18	bc2018--bc20...	97.9	25,923	6,775	Q38	175,644,772	121,687
Bio Sample 19	bc2019--bc20...	97.5	23,040	6,747	Q38	155,467,010	122,291
Bio Sample 20	bc2020--bc20...	97.9	26,138	6,646	Q38	173,730,992	121,650
Bio Sample 21	bc2021--bc20...	97.4	24,954	6,750	Q38	168,449,825	122,122
Bio Sample 22	bc2022--bc20...	97.9	22,402	6,627	Q38	148,478,815	121,233
Bio Sample 23	bc2023--bc20...	97.4	22,389	6,692	Q38	149,847,373	121,316
Bio Sample 24	bc2024--bc20...	97.8	24,244	6,677	Q38	161,888,862	121,594
Bio Sample 48	bc2048--bc20...	96.9	13,819	7,156	Q39	98,899,414	121,848
No Name	Not Barcoded	0.0	36,450	8,306	Q32	302,770,918	119,661

Recommended guidance for evaluating PureTarget repeat expansion panel sequencing run performance (cont.)

On-target coverage is affected by input gDNA quantity, input gDNA quality and multiplex level[1]

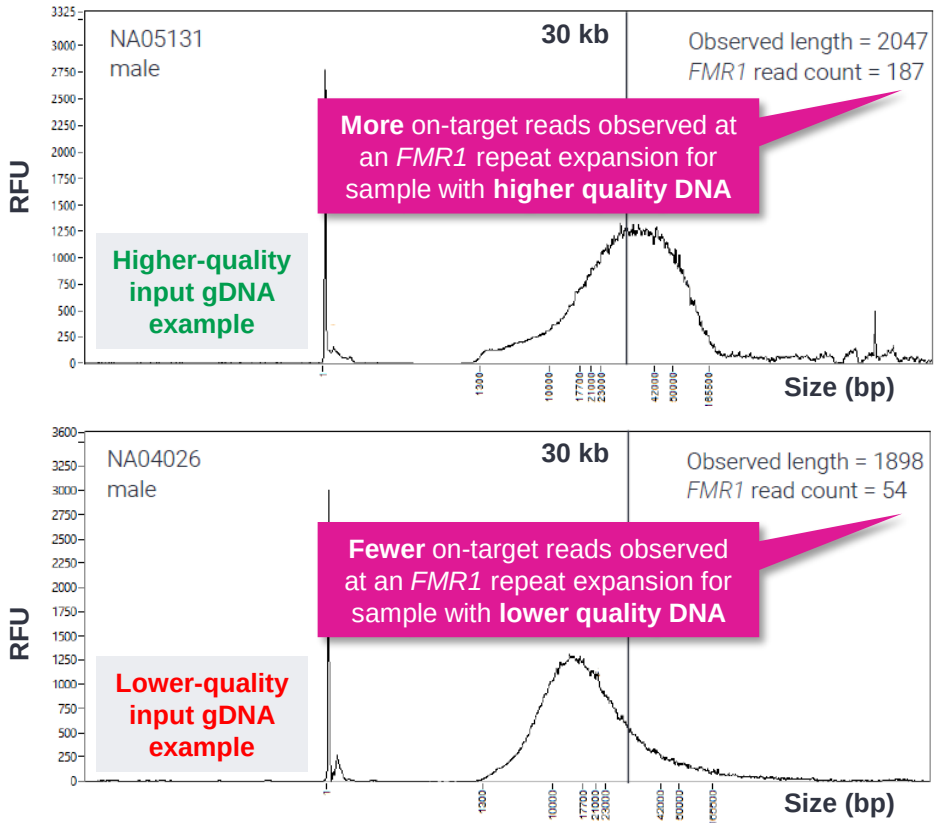
DNA Input Quantity vs. Coverage



DNA input quantity versus coverage. DNA was extracted from whole blood using the Nanobind PanDNA kit and run in an 8-plex.

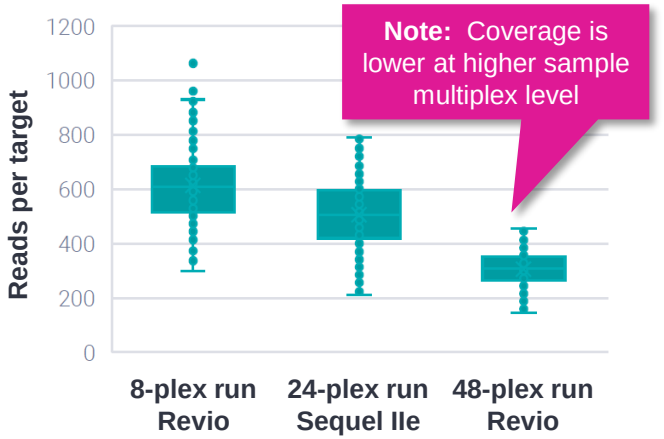
- PureTarget libraries do not use amplification to enrich targets but rather retain targets of interest and deplete off-target molecules
- As such, the library quantity loaded on the SMRT Cell is lower than other library types like WGS and the **sequencing yield for a sample can be influenced by how much starting DNA is used in the library prep**

Impact of Input Genomic DNA Quality on Coverage



- Comparison of a) high- and b) low-quality gDNA samples illustrate that more on-target reads are observed at an *FMR1* repeat expansion for the sample with higher molecular weight DNA

Sample Multiplex Level vs. Coverage



Sample multiplex level versus coverage. Samples were prepared from 2.0–2.5 µg of high molecular weight (HMW) DNA extracted with Nanobind PanDNA from whole blood.

- Deep coverage is achieved across 20 panel targets for 8-plex through 48-plex experimental designs

[1] See ***Application note — Comprehensive genotyping with the PureTarget repeat expansion panel and HiFi sequencing*** (102-326-614).

PureTarget data analysis workflow overview

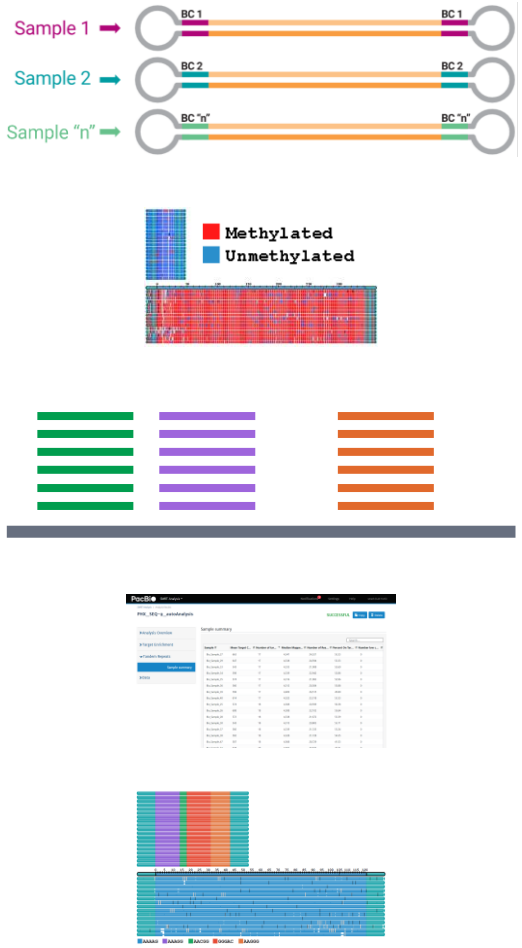
PureTarget data analysis workflow overview

Analysis of PureTarget repeat expansion libraries can be performed in SMRT Link using the PureTarget repeat expansion analysis workflow or at the command line[1]

SMRT Link PureTarget bioinformatics workflow[2]

SMRT Link

- Demultiplex `lima`
- 5mC probabilities
- Mapping `Pbmm2`
- Genotype `trgt`
- Visualize `TRVZ`



SMRT Link v13.1+

SMRT Analysis

Demultiplex

- Samples are demultiplexed using `lima`

5mC probabilities

- 5mC methylation probabilities for CpG sites

Mapping

- Reads are mapped to the hg38 reference genome with `pbmm2`

Genotype

- Repeat genotypes are called with `TRGT`

Visualize

- Visualizations are produced with `TRVZ`.

[1] See ***Application note – Analysis guide for PureTarget repeat expansion panel*** (102-326-616) for detailed descriptions of parameter settings for PureTarget analysis application.

[2] See ***SMRT Link User Guide*** (Documentation) for detailed descriptions of parameter settings for PureTarget analysis application.

PureTarget data analysis workflow overview (cont.)

File Downloads tab

Edit Output File Name Prefix **Example:**analysis-[multiple]-2928

File	Size	Type
Analysis Log		log
Input - Target BED file used in run	2 KB	unknown
QC - Target Genotype Table	68 KB	csv
SMRT Link Log	28 KB	log
TRGT - BAM (input for TRVZ)	14 MB	zip
TRGT - VCF files	194 KB	zip
TRVZ - all target plots (meth; allele)	39 MB	zip
TRVZ - all target plots (meth; waterfall)	38 MB	zip
TRVZ - all target plots (motifs; allele)	22 MB	zip
TRVZ - all target plots (motifs; waterfall)	26 MB	zip
Tandem Repeats	8 KB	JsonReport

- **QC – Target Genotype Table**
  - CSV file containing repeat unit sequence, allele count, min/max/consensus repeat array length, motif count, and motif span information

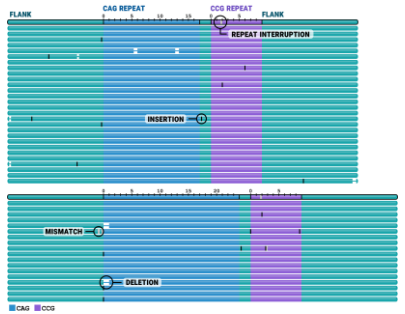
Sample	Bio_Sample_1
CNBP repeat unit	CAGG:CAGA:CA
CNBP read count allele 0	146
CNBP consensus size allele 0	139
CNBP min size allele 0	129
CNBP max size allele 0	317
CNBP motif counts allele 0	15_8_23
CNBP motif spans allele 0	0(0-61)_1(61-93)_2(93-139)

- **TRVZ - all target plots (meth, allele):**
  - Depicts consensus repeat alleles and reads aligning to them. Bases in repeats are colored by methylation levels.
- **TRVZ - all target plots (meth, waterfall):**
  - Depicts portions of reads spanning the repeat without aligning them, which is convenient for showing mosaicism. Bases in repeats are colored by methylation levels.
- **TRVZ - all target plots (motifs, allele):**
  - Depicts consensus repeat alleles and reads aligning to them. Bases in repeats are colored by repeat motif.
- **TRVZ - all target plots (motifs, waterfall):**
  - Depicts portions of reads spanning the repeat without aligning them, which is convenient for showing mosaicism. Bases in repeats are colored by repeat motif.

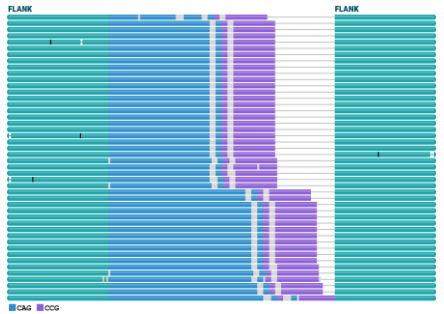
TRVZ is a companion visualization program for TRGT

- Create allele plots that depict repeat alleles and reads aligning to them
- Create waterfall plots that depict portions of HiFi reads spanning the repeat without aligning them; waterfall plots are especially convenient for visualizing mosaicism.
- Color code repeat structure on allele and waterfall plots
- Display 5mCpG methylation levels on allele and waterfall plots

Allele plot



Waterfall plot



[1] See GitHub documentation for detailed instructions on how to interpret the read pileup plots generated by TRVZ.

PacBio

Technical documentation & applications support resources

Technical resources for PureTarget library preparation, sequencing & data analysis

DNA sample preparation literature & other resources

- Brochure – Nanobind PanDNA kit (102-326-604)
- Procedure & checklist – Extracting HMW DNA from human whole blood using Nanobind kits (102-573-500)
- Procedure & checklist – Extracting HMW DNA from human whole blood with RBC lysis using Nanobind kits (103-377-500)
- Procedure & checklist – Extracting HMW DNA from cultured suspension cells using Nanobind kits (103-394-500)
- Procedure & checklist – Extracting HMW DNA from cultured adherent cells using Nanobind kits (102-573-600)

PureTarget library preparation literature & other resources

- Application note – Comprehensive genotyping with PureTarget repeat expansion panel and HiFi sequencing (102-326-614)
- Brochure – Comprehensive genotyping with PureTarget repeat expansion panel (102-326-609)
- Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400)
- Technical overview – PureTarget repeat expansion panel library preparation using PureTarget kit (103-418-100)

Data analysis resources

- Application note – Analysis guide for PureTarget repeat expansion panel (102-326-616)
- SMRT Link software installation guide [Link]
- SMRT Link user guide [Link]
- SMRT Tools reference guide [Link]

Technical resources for PureTarget library preparation, sequencing & data analysis (cont.)

Publications and posters

- ACMG 2024 Poster abstract - Fuligni, F. et al. (2024) Multiplex detection and quantification of neurological disease-associated repeat expansions using the PacBio Sequel IIe Platform. Genetics in Medicine Open. [DOI]

Example PacBio data sets

Application	Dataset	Data type	PacBio system
PureTarget repeat expansion panel	Repeat expansion panel Coriell samples [Link]	HiFi long read	Sequel IIe system
	Repeat expansion panel HG001 and HG002 48-plex [Link]	HiFi long read	Revio system
	Repeat expansion panel HG001 and HG002 8-plex [Link]	HiFi long read	Sequel IIe system

Appendix

Sample setup workflow overview for PureTarget libraries – Sequel II/IIe system

Follow sample setup instructions for PureTarget libraries in ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) – Do not use SMRT Link Sample Setup

1. Anneal sequencing primer
2. Bind sequencing polymerase
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

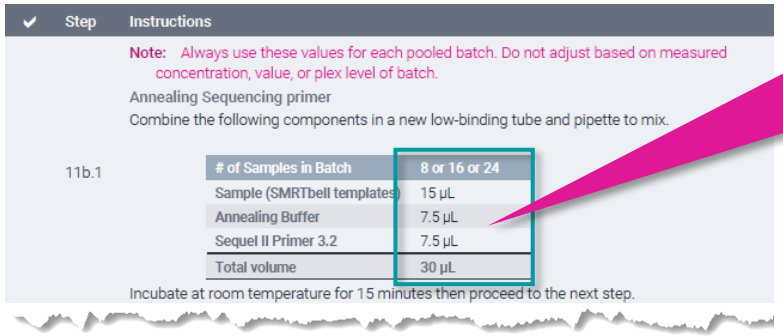
- Use entire volume (**15 µL**) of pooled SMRTbell templates per ABC reaction
- **Note:** Always use the fixed reagent volumes shown at each ABC step for each pooled batch

Sequel II/IIe system ABC workflow for PureTarget repeat expansion panel samples

8, 16 or 24 samples

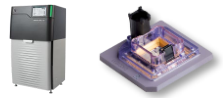
Follow **Procedure & checklist 103-329-400** for ABC instructions

ABC[1]
(Pool of 8/16/24 samples in 15 µL)



NOTE:
ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch



Sequence pooled samples on one Sequel II SMRT Cell

Sample setup procedure for PureTarget libraries – Sequel II/IIe system

Anneal sequencing primer

1 Anneal sequencing primer
↓
2 Bind sequencing polymerase
↓
3 Clean up SMRTbell complexes
↓ Safe stop
4 Internal control dilution
↓
5 Final loading dilution

Anneal sequencing primer

✔	Step	Instructions
		Note: Always use these values for each pooled batch. Do not adjust based on measured concentration, value, or plex level of batch.
		Annealing Sequencing primer
		Combine the following components in a new low-binding tube and pipette to mix.
	11b.1	

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

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

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

Sample setup procedure for PureTarget libraries – Sequel II/IIe system (cont.)

Bind sequencing polymerase

1 Anneal sequencing primer
↓
2 Bind sequencing polymerase
↓
3 Clean up SMRTbell complexes
↓ Safe stop
4 Internal control dilution
↓
5 Final loading dilution

Dilute sequencing polymerase

✔	Step	Instructions
		Combine the following components in a single low-bind tube and pipette to mix. The prepared volume of diluted polymerase is sufficient to process all specified samples in a batch.
	11b.2	

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

Diluted polymerase must be used immediately.

- **Note:** For PureTarget repeat expansion samples, polymerase working solution concentration after performing polymerase dilution step is **higher** than for Kinnex & WGS samples

Bind sequencing polymerase

✔	Step	Instructions
		Add annealed sample to diluted polymerase and finger tap or pipette to mix.
	11b.3	

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

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

NOTE:
ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

Sample setup procedure for PureTarget libraries – Sequel II/IIe system (cont.)

Clean up SMRTbell complexes

1 Anneal sequencing primer

↓

2 Bind sequencing polymerase

↓

3 Clean up SMRTbell complexes

↓ Safe stop

4 Internal control dilution

↓

5 Final loading dilution

Purification of Polymerase Bound SMRTbell Complexes

✔	Step	Instructions

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

# of Samples in Batch	8 or 16 or 24
Binding reaction	60 µL
ABC buffer	40 µL
Total Volume	100 µL

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

# of Samples in Batch	8 or 16 or 24
Binding reaction	100 µL
Cleanup beads	120 µL
Total Volume	220 µL

11b.4

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

# of Samples in Batch	8 or 16 or 24
Sequel II loading buffer 3.2	50 µL

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

- Equilibrate SMRTbell cleanup beads and loading buffer to room temperature at least 30 min before use

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

- **Do not** wash bead pellet with ethanol

Sample setup procedure for PureTarget libraries – Sequel II/IIe system (cont.)

Internal control dilution

1. Anneal sequencing primer
2. Bind sequencing polymerase
3. Clean up SMRTbell complexes
   - Safe stop
4. Internal control dilution
5. Final loading dilution

Internal control dilution – First dilution

✔	Step	Instructions
	11b.5	1st Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

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

- **Prepare only <u>one</u> control dilution reaction**, regardless of number of samples (up to 24 samples per SMRT Cell for Sequel II/IIe system)

Internal control dilution – Second dilution

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

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

Internal control dilution – Third dilution

✔	Step	Instructions
	11b.7	3rd Dilution. Mix well by flicking the tube by hand and pulse-spin to collect contents and keep on ice.

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

Sample setup procedure for PureTarget libraries – Sequel II/IIe system (cont.)

Final loading dilution

1 Anneal sequencing primer

↓

2 Bind sequencing polymerase

↓

3 Clean up SMRTbell complexes

↓ Safe stop

4 Internal control dilution

↓

5 Final loading dilution

Final loading dilution

✔	Step	Instructions
		Combine the following and protect from light:
	11b.8	

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

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

PROTOCOL COMPLETE

- Add **50 µL Prepared sample + 67 Sequel II loading buffer 3.2 + 3 µL diluted internal control**
  - → Transfer entire mixture (**100 µL**) to appropriate sample well in Revio sequencing plate

NOTE:

ALWAYS USE THESE FIXED VOLUMES FOR EACH POOLED BATCH

→ **Do not adjust reagent volumes** based on measured sample concentration value or plex level of batch

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