Technical overview
Revio system v13.3 + SPRQ chemistry

Instrument control software, SMRT Link software & applications updates for the Revio system with SPRQ chemistry

PN 103-585-900 Rev 01 | December 2024

Technical overview

Revio system v13.3 + SPRQ chemistry

Instrument control software, SMRT Link software & applications updates for the Revio system with SPRQ chemistry

1. Revio system v13.3 key features & benefits overview
2. Revio system v13.3 SPRQ consumables & ICS
3. Revio system v13.3 user experience improvements
4. Revio system v13.3 applications & protocol updates
5. Revio system v13.3 example sequencing performance
6. Technical documentation & applications support resources
7. Appendix

PacBio

Revio system v13.3
key features & benefits

What is staying the same in Revio system v13.3?

No major changes to core HiFi library prep kits and Revio hardware / Revio SMRT Cells / Revio data file formats

HiFi library prep kits

SMRTbell prep kit 3.0 / HiFi prep kit 96[1] / HiFi plex prep kit 96 / Kinnex / PureTarget

Revio instrument hardware

Four independent stages with multiple movie time options

Revio SMRT Cell

High-density ZMW SMRT Cell

Revio data file formats

hifi_reads.bam + fail_reads.bam

[1] Updated Revio SPRQ HiFi prep kit 96 **bundle** PN 103-522-600 includes Revio SPRQ polymerase kit 96, HiFi prep kit 96, SMRTbell cleanup beads and other reagents to support high-throughput sample processing.

What is new in Revio system v13.3?

New Revio system v13.3 with SPRQ chemistry makes HiFi sequencing easier and more cost-effective

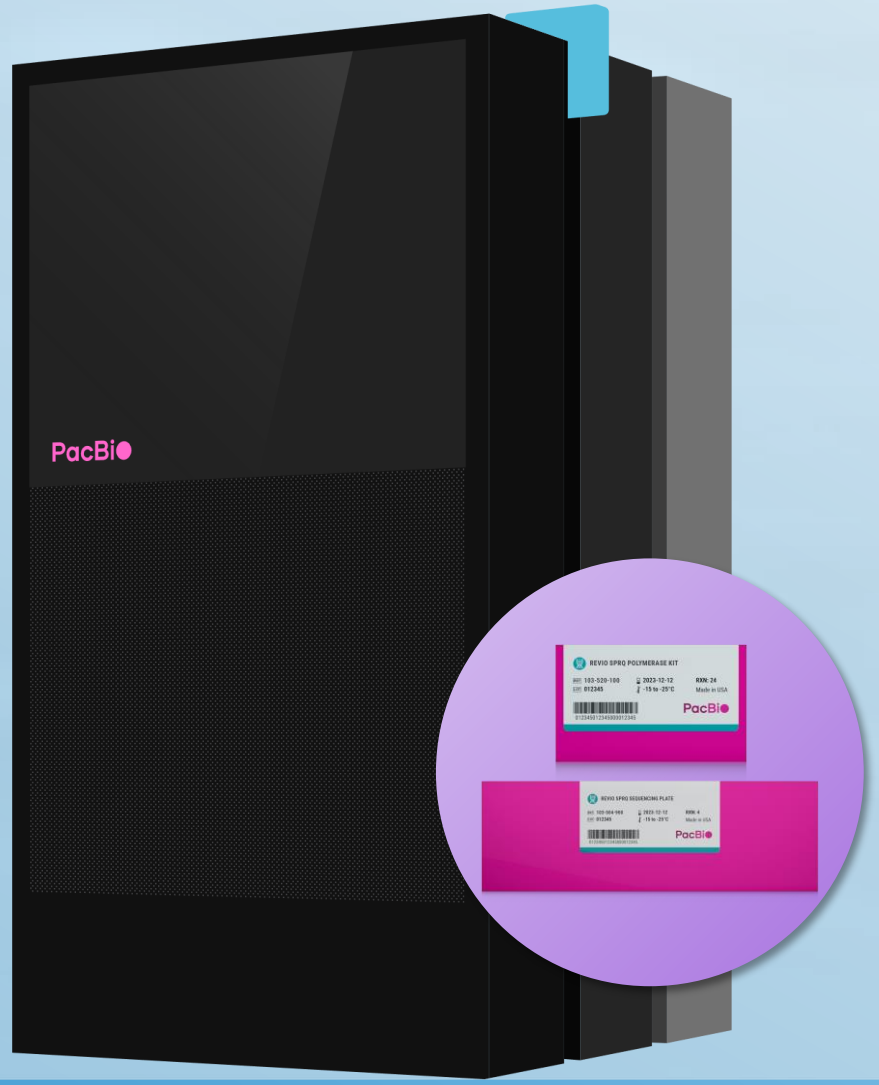
More data
480 Gb HiFi data per run (4 Revio SMRT Cells) or
120 Gb per Revio SMRT Cell

More sample types
4-fold reduction in DNA input requirements enables analysis of more precious sample types

More accuracy
Improved 5mC calling performance in CpG contexts enables accurate methylation profiles

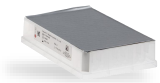
More robust
Chemistry improvements enable more consistent sequencing performance for small inserts (≥1 kb)

More streamlined
New automated on-instrument 6mA calling, SMRT Link user experience improvements and support for SMRT Link Cloud[1]



[1] SMRT Link Cloud features includes instrument management, sample setup, run design, and run QC reports (SMRT Link Cloud does not include any analysis module).

Revio system v13.3 key workflow updates

Revio workflow step	What's new	What stays the same
DNA sample extraction	  • New saliva HMW DNA extraction procedure using Genotek Oragene collection devices and Nanobind kits	• Existing Nanobind HMW DNA extraction workflows • Existing Nanobind kits
SMRTbell library preparation	  • Reduced genomic DNA input requirements for HiFi WGS library preparation + re-optimized SRE & shearing conditions to support low DNA input amounts (500 ng)	• Core SMRTbell library construction workflow • Existing HiFi library preparation kits • Existing applications support
Sample setup (ABC)	  • Updated sample setup ABC (annealing / binding / complex cleanup) workflow guidance for new Revio SPRQ polymerase kit • SMRT Link v25.1 Sample Setup Loading Calculator support for new Revio SPRQ polymerase kit • SMRT Link 25.1 Sample Setup GUI user experience improvements	• Overall Revio sample setup workflow • Existing applications support
Run design	  • SMRT Link v25.1 run design support for new Revio SPRQ sequencing plate • SMRT Link v25.1 run design GUI user experience improvements	• Overall Revio run design workflow • Existing applications support
Sequencing	 • ICS v13.3 support for new Revio SPRQ chemistry • ICS v13.3 support for reduced DNA input requirements • ICS v13.3 support for improved 5mc CpG calling and on-instrument 6mA calling for Fiber-Seq assays	• Overall Revio run setup workflow • Existing Revio SMRT Cell • Existing Revio on-instrument analysis features

Revio system v13.3 key software updates

New ICS and SMRT Link software enables support for Revio SPRQ chemistry

Revio system supported software			
	ICS v13.3[1]		SMRT Link v25.1[2]
Instrument control	• Minor ICS software bug fixes • Improved sequencing workflow to support reduced DNA input amounts	Instruments	• General user interface improvements
Primary and post-primary analysis	• Added support for new Revio SPRQ sequencing chemistry[3]	Sample Setup	• Loading calculator support for new Revio SPRQ polymerase kit[3]
On-instrument CCS analysis	• Added support for new SPRQ sequencing chemistry[3]	Runs	• Run design support for new Revio SPRQ sequencing plate[3] • General user interface improvements
On-instrument methylation calling	• Improved accuracy and increased confidence of 5mCpG calling • On-instrument 6mA caller for Fiber-seq chromatin assay	Data Management	No major changes
On-instrument barcode demux	No major changes	SMRT Analysis	• General usability and user experience improvements

1 ICS v13.3 is only available for Revio systems and does not support Sequel II/IIe systems.
2 SMRT Link v25.1 supports Revio system ICS v13.3 and Vega system ICS v1.0. SMRT Link v25.1 does not support Sequel II/IIe systems
3 ICS v13.3 also retains support for original Revio sequencing chemistry.

Revio system v13.3 key software updates (cont.)

Revio v13.3 with SPRQ chemistry SMRT Link compatible versions

SMRT Link
v25.1[1]

SMRT Link Lite
v25.1

SMRT Link Cloud
v25.1[1,2]

SMRT Link product features

Feature	SMRT Link	SMRT Link Lite	SMRT Link Cloud
Instrument management	Y	Y	Y
Run setup and QC	Y	Y	Y
Push-button secondary analysis (SMRT Analysis)	Y	N	N
3rd party and PacBio Compatible Partner analysis	Y	Y	Y
API access	Y	Y	N

1 SMRT Link v25.1 and SMRT Link Cloud do not support Sequel II/IIe systems.
2 SMRT Link Cloud is a PacBio-hosted and managed private workspace that provides support for instrument management, Sample Setup, Runs, and QC but doe not feature any analysis capabilities (i.e., no SMRT Analysis module). See **Application brief – SMRT Link** (102-326-628).

Revio system v13.3 SPRQ consumables & instrument control software

Revio system v13.3 release includes new polymerase kit, sequencing plate & ICS

New consumables and instrument control software enable an improved user experience for HiFi sequencing

Revio SPRQ sequencing plate

4 rxn – PN 103-504-900
1 rxn – PN 103-512-700[1]

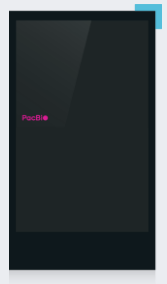
New SPRQ chemistry formulation improves sample loading efficiency on Revio SMRT Cells

Revio SPRQ polymerase kit

24 rxn – PN 103-520-100[2]
96 rxn – PN 103-522-600[3]

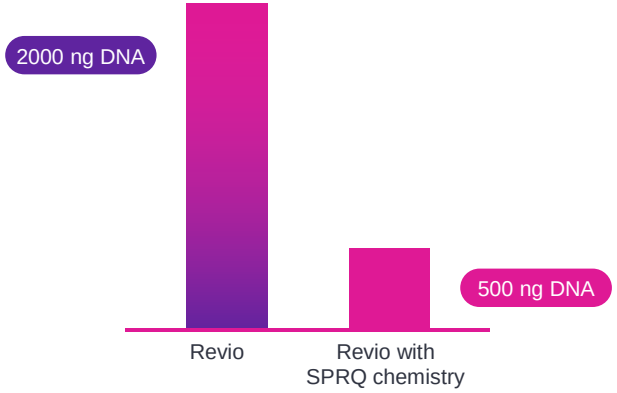
New SPRQ polymerase enzyme and reagents improve HiFi sequencing performance

Revio ICS v13.3

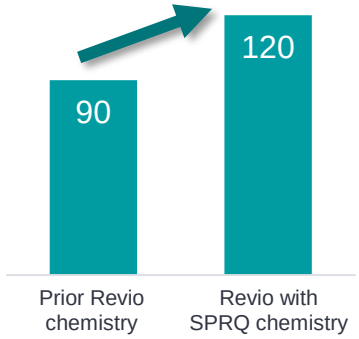


ICS v13.3 improves 5mC calling accuracy and features new on-instrument 6mA calling for Fiber-Seq assays

4× lower DNA input for library prep



33% increase in HiFi yield / SMRT Cell



HiFi data (Gb)

New & improved multi-omics capabilities

PacBio

1 Revio SPRQ sequencing plate – 1 rxn part is listed in Customer price list and can be ordered through standard purchasing processes and the customer hub (E-commerce).
2 Revio SPRQ polymerase kit + cleanup beads bundle PN 103-520-100 includes Revio SPRQ polymerase kit and SMRTbell cleanup beads.
3 Revio SPRQ HiFi prep kit 96 bundle PN 103-522-600 includes Revio SPRQ polymerase kit 96, HiFi prep kit 96, SMRTbell cleanup beads and other reagents to support high-throughput sample processing.

Revio system v13.3 consumable compatibility

Revio SPRQ polymerase kit & Revio SPRQ sequencing plate must be used together and cannot be combined with older Revio polymerase kit & Revio sequencing plate consumables

V13.1 ICS

Compatible

- Revio polymerase kit
  Revio sequencing plate

Not compatible

- ~~Revio SPRQ polymerase kit~~
  ~~Revio SPRQ sequencing plate~~

V13.3 ICS

Cannot mix and match SPRQ and non-SPRQ reagents in the same run

Compatible

- Revio polymerase kit
  Revio sequencing plate
- Revio SPRQ polymerase kit
  Revio SPRQ sequencing plate

Not compatible

- ~~Revio SPRQ polymerase kit~~
  ~~Revio sequencing plate~~
- ~~Revio polymerase kit~~
  ~~Revio SPRQ sequencing plate~~

New Revio SPRQ polymerase kit (cont.)

Revio polymerase kit configuration and layout comparison

Revio SPRQ polymerase kit

(Supports up to 24 binding Rxs / 24 Revio SMRT Cells)

#	Component	Part number	Qty	Color	Volume
1	Annealing buffer	102-797-600	1	light blue	530 µL
2	Standard sequencing primer	102-797-700	1	light green	530 µL
3	Kinnex™ sequencing primer	103-179-000	1	light purple	530 µL
4-5	Polymerase buffer	102-797-800	2	yellow	1.2 mL
6	Sequencing polymerase	103-512-800	1	purple	102 µL
7-9	Dilution buffer	102-797-900	3	blue	1.7 mL
10	Sequencing control	103-508-800	1	red	27 µL
11	Loading buffer	103-485-400	1	green	1.46 mL

Note: Revio SPRQ polymerase kit contains **new** reagents with **new** part numbers – but **existing tube labels (tube names) stay the same**

NEW SPRQ enzyme and part number

NEW DNA control complex and part number

NEW loading buffer and part number

Revio polymerase kit

(Supports up to 24 binding Rxs / 24 Revio SMRT Cells)

#	Component	Part number	Qty	Color	Volume
1	Annealing buffer	102-797-600	1	light blue	530 µL
2	Standard sequencing primer	102-797-700	1	light green	530 µL
3	Kinnex™ sequencing primer	103-179-000	1	light purple	530 µL
4-5	Polymerase buffer	102-797-800	2	yellow	1.2 mL
6	Sequencing polymerase	102-797-300	1	purple	102 µL
7-9	Dilution buffer	102-797-900	3	blue	1.7 mL
10	Sequencing control	102-798-000	1	red	27 µL
11-12	Loading buffer	102-797-500	2	green	1.2 mL

11 tubes

FEWER tubes (only 1 Loading buffer tube)

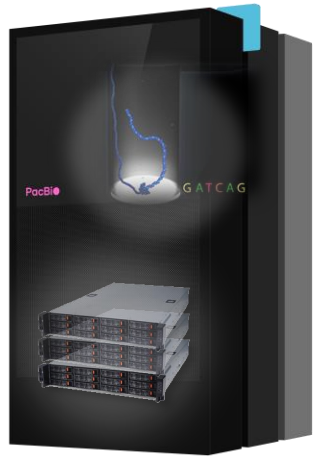
12 tubes

New Revio SPRQ chemistry improves HiFi data yield

New SPRQ polymerase enzyme enables more efficient generation of HiFi data compared to previous chemistry

Revio SPRQ chemistry enables HiFi sequencing to generate **~33% higher HiFi data yields** compared to prior chemistry[1]

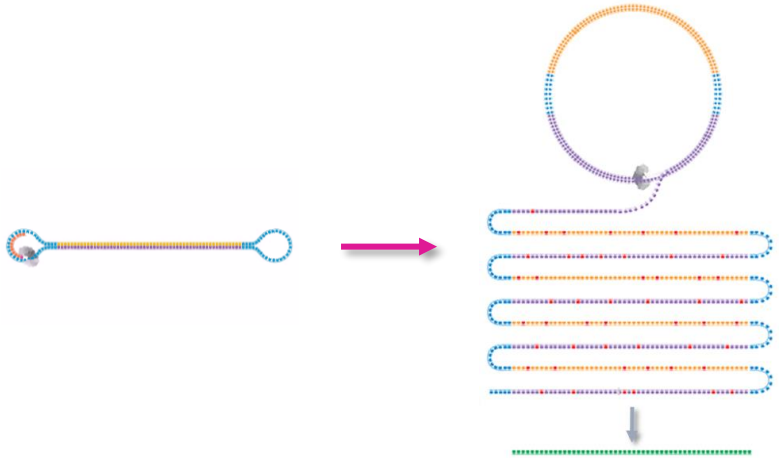
Revio SPRQ polymerase kit



Revio system v13.3

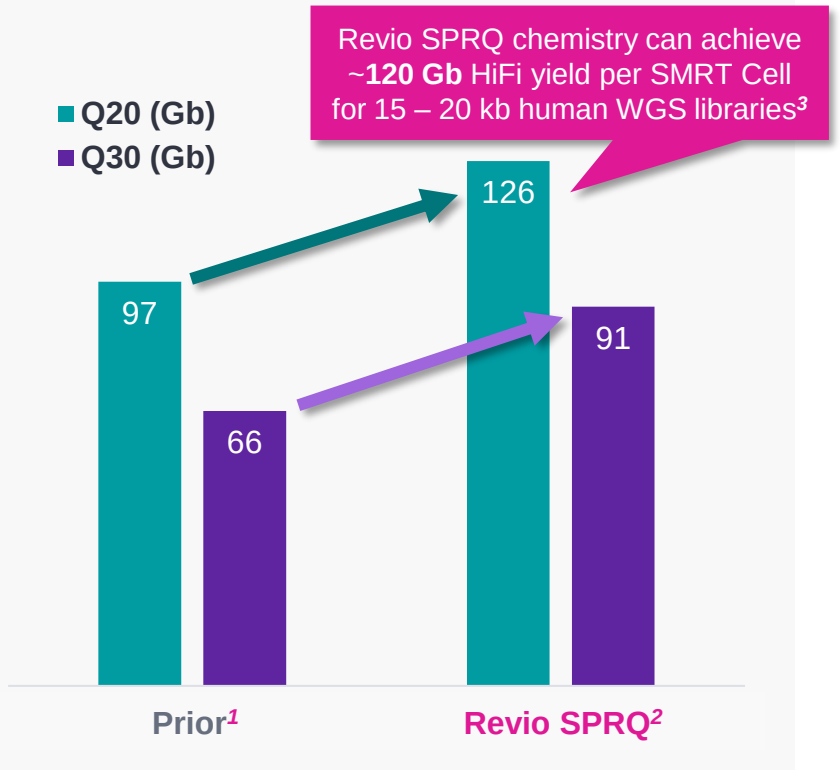
SPRQ polymerase enzyme features **improved processivity**

- Reduces number of early-terminating reads and generates a higher proportion of reads that are long enough to make multiple passes around SMRTbell template
  - → Enables **overall higher *P1*-to-HiFi (≥Q20 CCS) read conversion rate and thus higher HiFi data yield**



HiFi read

QV and HiFi data yield



1 Example HiFi data shown for a human HG002 sample run using Revio polymerase kit and Revio sequencing plate chemistry.
2 Example HiFi data shown for a human HG002 sample run using Revio SPRQ polymerase kit with Revio SPRQ sequencing plate chemistry.
3 HiFi yield is dependent on library quality, library insert size and sequencing preparation procedures. Specified yield is based on high-quality samples prepared following best practices.

New Revio SPRQ DNA sequencing control performance

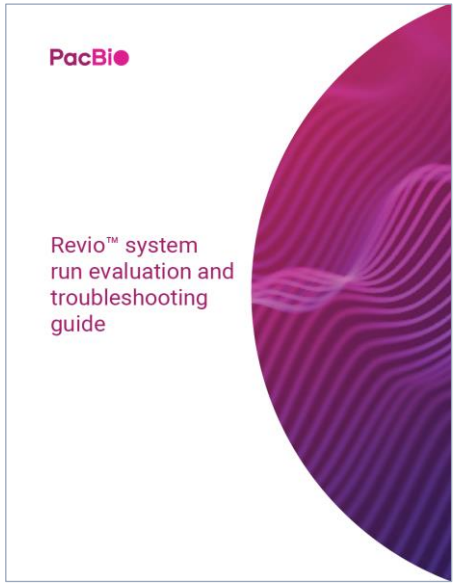
SPRQ DNA sequencing control is bound to new Revio SPRQ polymerase enzyme

Metric	Expected control performance[1]	
	Revio v13.1 DNA control	Revio v13.3 SPRQ DNA control
Control read count	≥500	≥500
Control polymerase read length (Mean)	≥40 kb	≥50 kb
Control concordance (Mean)	≥0.88	≥0.88

[1] Expected control performance metrics shown are based on a 24 hrs movie collection time.

DNA internal control metrics are useful to help assess the performance of a sequencing run

- If control read count or control read length is lower than expected
  - → Focus troubleshooting on PacBio system and/or consumables
- If control performance appears normal,
  - → Focus troubleshooting on investigating DNA sample quality and/or library prep QC metrics
- For sequencing performance troubleshooting guidance, refer to ***Revio run evaluation and troubleshooting guide*** (103-380-300)[2] or contact PacBio Technical Support.



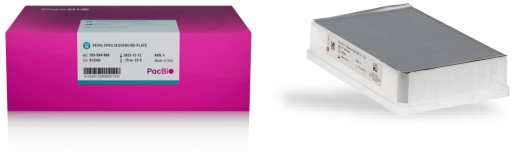
[2] See ***Revio run evaluation and troubleshooting guide*** (103-380-300) for general information about troubleshooting SMRT sequencing performance issues.

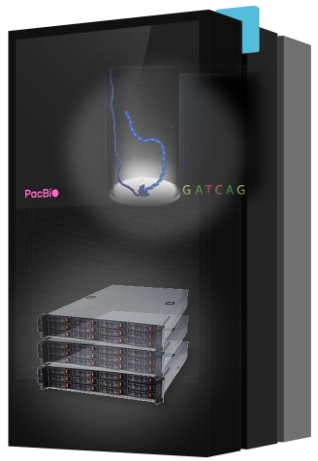
New Revio SPRQ chemistry reduces DNA input requirements

New SPRQ sequencing plate improves sample loading efficiency on Revio SMRT Cells, enabling 4× reduction in DNA sample input requirements for library prep compared to previous chemistry

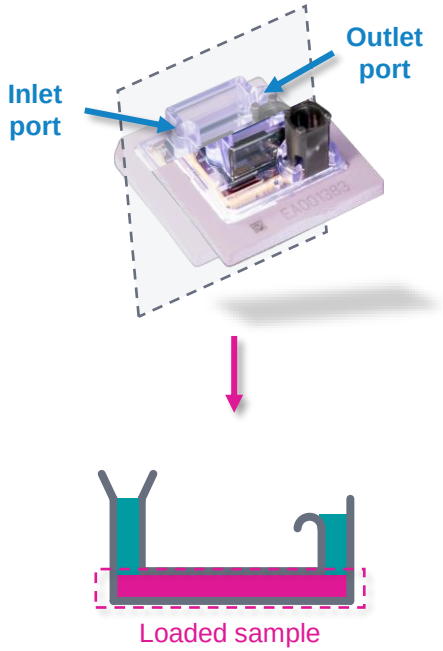
Revio SPRQ chemistry enables HiFi sequencing to be performed with precious genomic samples using as little as **500 ng** of input DNA

Revio SPRQ sequencing plate

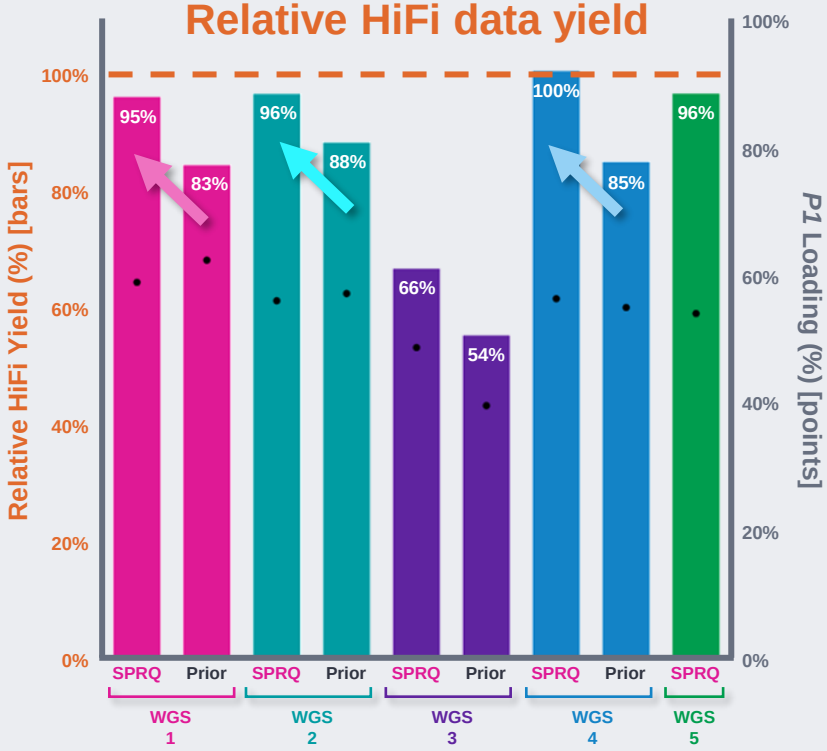




Revio system v13.3



New SPRQ sequencing plate chemistry & ICS v13.3 robotic fluidic handling workflow **improve sample loading efficiency and reduce bulk sample waste**



Experiment conditions[1]

SPRQ

500 ng DNA input for WGS library prep

Pre-release **Revio SPRQ sequencing plate chemistry**

Revio non-SPRQ polymerase kit

Prior

2000 ng DNA input for WGS library prep

Prior Revio sequencing plate chemistry

Revio non-SPRQ polymerase kit

WGS libraries run with SPRQ sequencing plate chemistry show **comparable or improved HiFi yields** over prior chemistry while using **4× less DNA input** for library prep & sequencing

PacBio

[1] HiFi data shown for Revio SPRQ sequencing plate chemistry were generated with a Revio (non-SPRQ) polymerase kit in conjunction with use of reduced input DNA amounts (**500 ng**) for WGS library prep. Data shown for prior Revio sequencing plate chemistry were generated with a Revio (non-SPRQ) polymerase kit in conjunction with use of higher input DNA amounts (**2000 ng**) for WGS library prep.

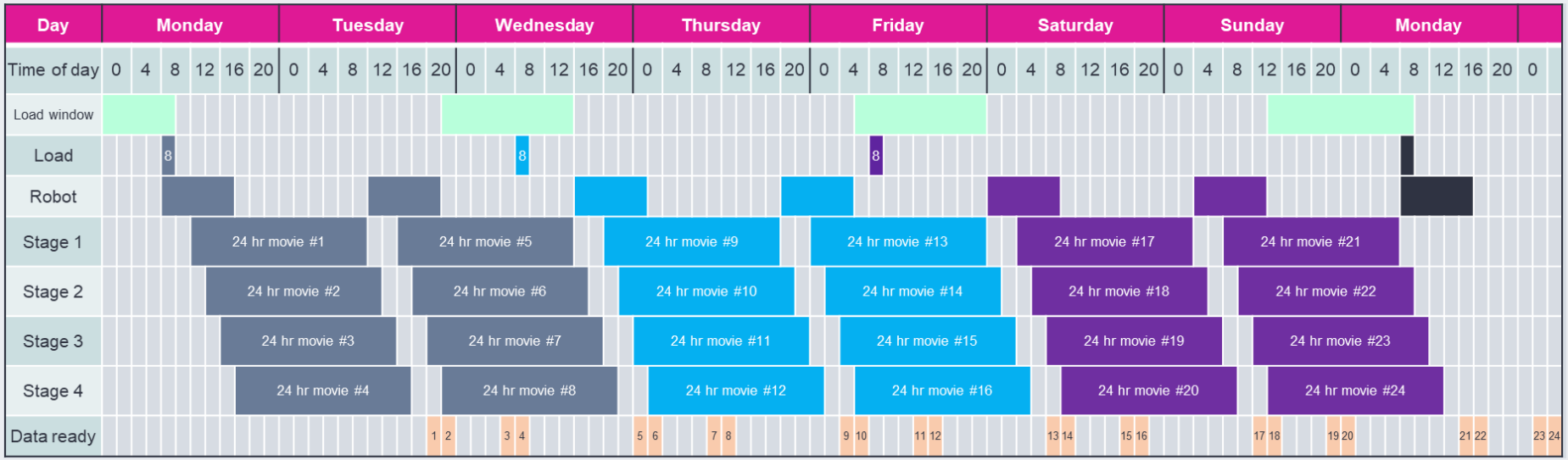
Revio instrument robotic workflow run times remain the same in v13.3 vs. v13.1

No modifications required to existing high-utilization Revio production run schedules

Example Revio system run schedule to process 24 Revio SMRT Cells per week[1]

Example Revio ICS v13.1 / 13.3 run schedule with 24-hour movies, 8 SMRT Cells per run

- Default run conditions, incl. adaptive loading
- 24 SMRT Cells / week
- Load same time of day on Mon, Wed, Fri (3 touch points)



Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 576 hours of automated sequencing runtime per week (144 hours × 4 stages) and 3 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

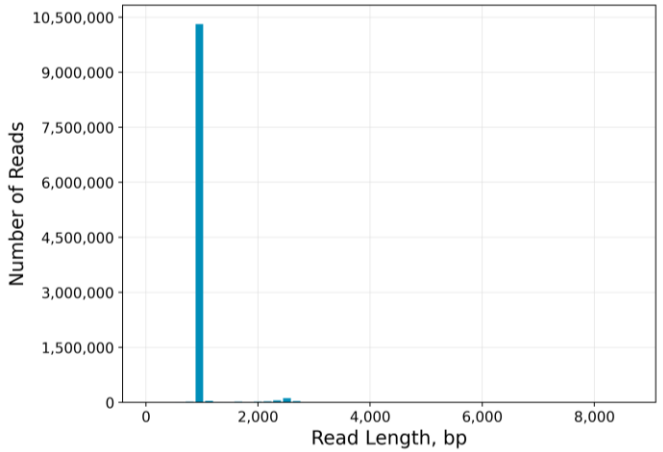
[2] Refer to **Appendix** for more examples of Revio production run schedules for different movie times and different throughput requirements.

New Revio SPRQ chemistry enables more robust sequencing performance for short inserts

New SPRQ chemistry improves consistency of loading short insert libraries on Revio SMRT Cells[1]

1 kb Amplicon

HiFi read length distribution

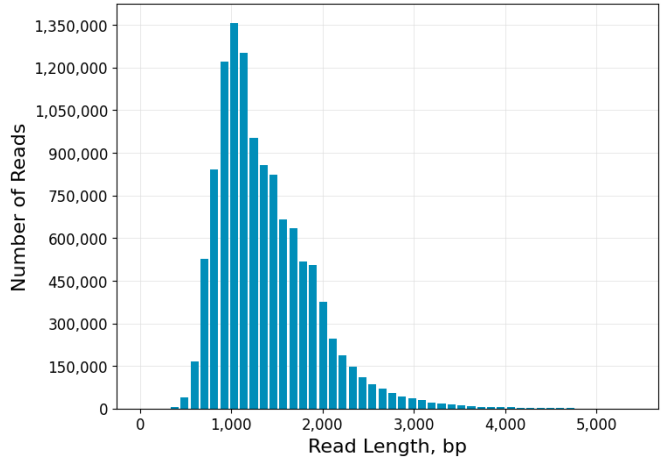


P1	60.7%
HiFi Reads	10.7 M
HiFi Base Yield	11.2 Gb
Mean HiFi Read Length	1.04 kb
Median HiFi Read Quality	Q60
HiFi Read Mean # of Passes	46

Example sequencing metrics for 1 kb amplicon sample run with Revio SPRQ sequencing plate / 200 pM on-plate concentration / 24-hrs movie time. HiFi yields ranged from ~10 – 12 M HiFi reads per Revio SMRT Cell for *P1* loadings ~60 – 75%.

1.3 kb Monomer full-length RNA

HiFi read length distribution

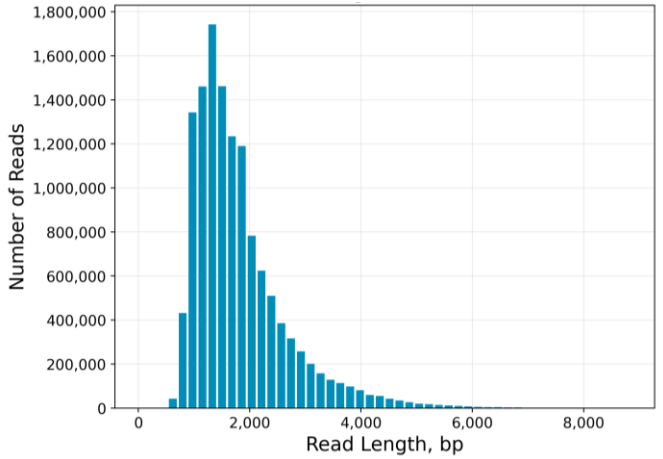


P1	74%
HiFi Reads	11.9 M
HiFi Base Yield	16.2 Gb
Mean HiFi Read Length	1.37 kb
Median HiFi Read Quality	Q48
HiFi Read Mean # of Passes	39

Example sequencing metrics for 1.3 kb monomer (non-Kinnex) full-length RNA sample run with Revio SPRQ sequencing plate / 200 pM on-plate concentration / 24-hrs movie time. HiFi yields ranged from ~12 – 13 M HiFi reads per Revio SMRT Cell for *P1* loadings ~70 – 80%.

1.8 kb Monomer full-length RNA

HiFi read length distribution



P1	82%
HiFi Reads	12.9 M
HiFi Base Yield	23.2 Gb
Mean HiFi Read Length	1.8 kb
Median HiFi Read Quality	Q45
HiFi Read Mean # of Passes	33

Example sequencing metrics for 1.8 kb UHRR monomer (non-Kinnex) full-length RNA sample run with Revio SPRQ sequencing plate / 200 pM on-plate concentration / 24-hrs movie time. HiFi yields ranged from ~11 – 13 M HiFi reads per Revio SMRT Cell for *P1* loadings ~65 – 85%.

[1] Recommended on-plate loading concentration range for amplicon library samples run with SPRQ chemistry is 200 – 300 pM.

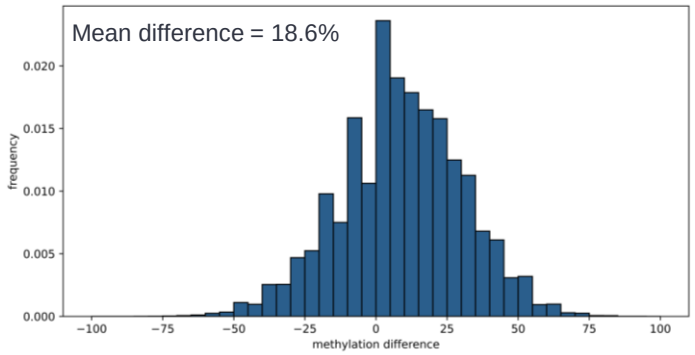
Revio system v13.3 improves on-instrument methylation detection capabilities

Updated on-instrument methylation analysis software improves 5mC calling performance[1]

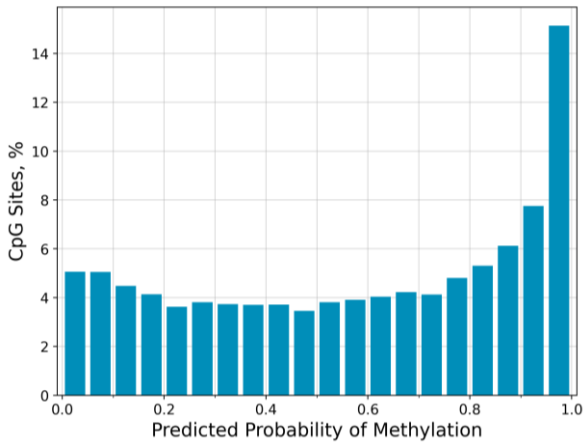
Example HG002 results for Revio ICS v13.1 / SMRT Link v13.1

5mC (CpG)	Accuracy[3]
jasmine v2.0	82%

5mC calling difference to WGS bisulfite sequencing



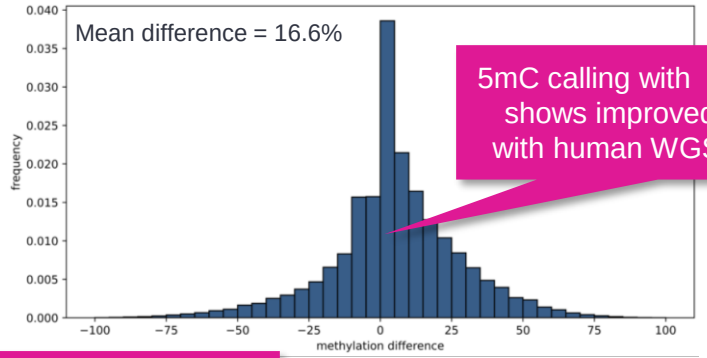
Predicted probability of 5mC methylation in CpG sites



Example HG002 results for Revio ICS v13.3[2]

5mC (CpG)	Accuracy[3]
jasmine v2.4	93%

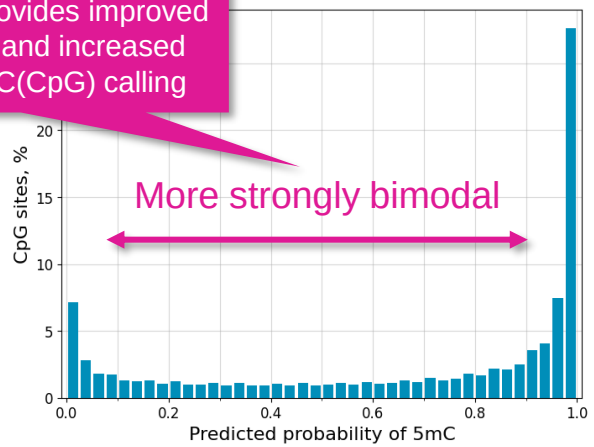
5mC calling difference to WGS bisulfite sequencing



5mC calling with Revio ICS v13.3 shows improved concordance with human WGS bisulfite data[4]

Revio ICS v13.3 provides improved accuracy (+10%) and increased confidence of 5mC(CpG) calling

Predicted probability of 5mC methylation in CpG sites



1 Revio system v13.3 methylation calling is automatically run for all samples and improves 5mC (in CPG) calling performance for both Revio SPRQ chemistry as well as the prior Revio v13.1 chemistry.
2 5mC calling feature is only available on-instrument in Revio system ICS v13.3 and is not available for off-instrument analysis in SMRT Link v25.1 user interface under Data Utilities. If needed, however, users can still use the command line tool version of jasmine available through the PacBio GitHub website at https://github.com/PacificBiosciences/jasmine
3 Example HG002 5mC (CpG) methylation calling data are shown. Accuracy = (TP + TN) / (TP + TN + FP + FN).
4 Methylation difference plots were generated by comparing Revio system methylation calling data against human WGS bisulfite data.

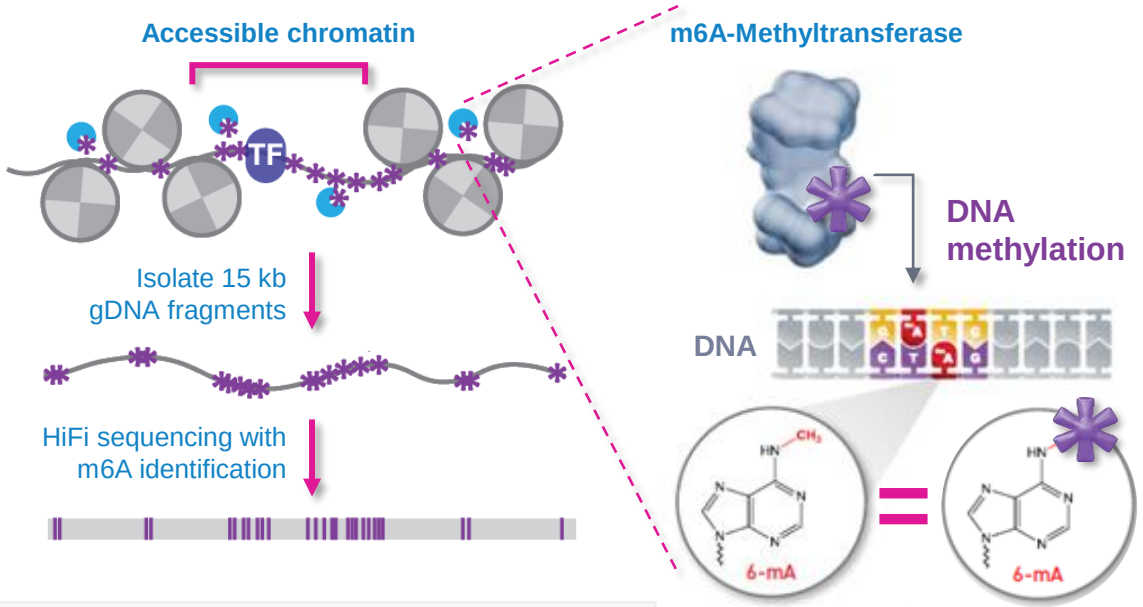
Revio system v13.3 improves on-instrument methylation detection capabilities

Updated on-instrument methylation analysis software supports 6mA calling to enable Fiber-seq[1] applications

NEW Revio ICS v13.3 on-instrument 6mA calling feature

- On-instrument 6mA calling feature is intended to support enablement of Fiber-Seq applications and is **not** intended for use as a general 6mA caller for microbial genome analysis applications[2]

Single-molecule chromatin fiber sequencing



6mA calling is automatically performed on all samples for all Revio chemistries

6mA methylation calling facilitates Fiber-Seq analyses

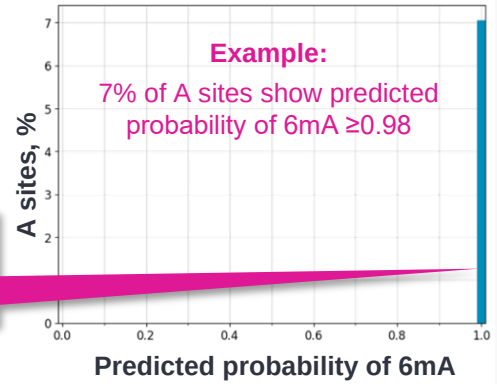
On-instrument 6mA calling

Modification	Motif	Scored sites	Modified sites (Pr > 0.5)
6mA	A	7.0%	7.0%

View 6mA calling results in SMRT Link

Data Management

6mA report typically displays a single peak[3]

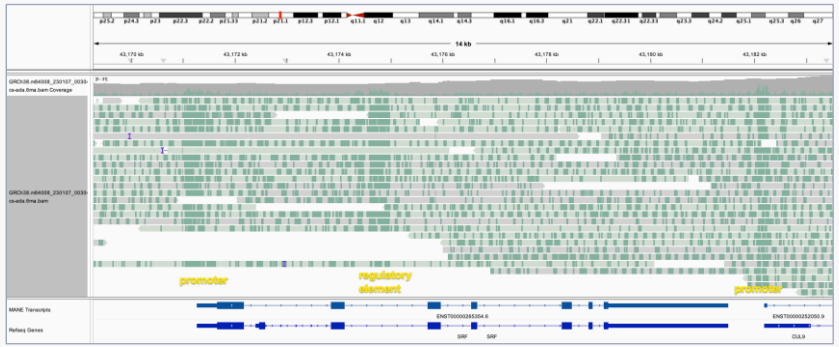


Example results for HG002 are shown. Revio on-instrument calling has high accuracy for 6mA, equivalent to Fibertools for Fiber-seq assays.

igv

Non-methylated bases

m6A marked bases



1 Stergachis, A., et al. (2022). Single-molecule regulatory architectures captured by chromatin fiber sequencing. Science 368, 1449-1454.
2 **Note:** For microbial 6mA and 4mC base modification detection applications, specify to save kinetic information in Run Design and then perform 6mA and 4mC calling in SMRT Link.
3 For on-instrument 6mA analysis in Revio system ICS v13.3, the model only reports 6mAs where probability of modification is >= 0.98 in order to reduce FPs and maximize disk use efficiency

Revio system v13.3 output files & directory structure remain unchanged from v13.1

Example Revio v13.3 output file directory structures[1] for non-indexed samples vs. indexed (barcoded) samples

Revio system v13.3 (Non-indexed samples)

```
r84001_20221122_134701
└── 1_A01
    ├── fail_reads
    │   ├── m84001_250101_123456_s1.fail_reads.bam
    │   └── m84001_250101_123456_s1.fail_reads.bam.pbi
    ├── hifi_reads
    │   ├── m84001_250101_123456_s1.hifi_reads.bam
    │   └── m84001_250101_123456_s1.hifi_reads.bam.pbi
    ├── metadata
    ├── pb_formats
    └── statistics
        └── m84001_250101_123456_s1.report.pdf
```

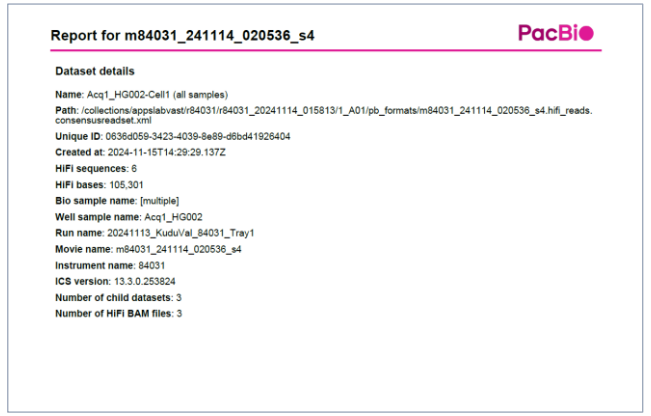
Average `hifi_reads.bam` file size per SMRT Cell is ~60 GB with SPRQ chemistry (includes 5mC and 6mA data)

An **on-instrument dataset report (PDF)** is automatically generated and saved in **statistics** folder and may be reviewed for troubleshooting purposes

Revio system v13.3 (Indexed samples)

```
r84001_20221122_134701
└── 1_A01
    ├── fail_reads
    │   ├── m84001_250101_123456_s1.fail_reads.bc2001.bam
    │   ├── m84001_250101_123456_s1.fail_reads.bc2001.bam.pbi
    │   ├── m84001_250101_123456_s1.fail_reads.bc2002.bam
    │   ├── m84001_250101_123456_s1.fail_reads.bc2002.bam.pbi
    │   ├── m84001_250101_123456_s1.fail_reads.bc2003.bam
    │   ├── m84001_250101_123456_s1.fail_reads.bc2003.bam.pbi
    │   ├── m84001_250101_123456_s1.fail_reads.unassigned.bam
    │   └── m84001_250101_123456_s1.fail_reads.unassigned.bam.pbi
    ├── hifi_reads
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2001.bam
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2001.bam.pbi
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2002.bam
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2002.bam.pbi
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2003.bam
    │   ├── m84001_250101_123456_s1.hifi_reads.bc2003.bam.pbi
    │   ├── m84001_250101_123456_s1.hifi_reads.unassigned.bam
    │   └── m84001_250101_123456_s1.hifi_reads.unassigned.bam.pbi
    ├── metadata
    ├── pb_formats
    └── statistics
        └── m84001_250101_123456_s1.report.pdf
```

On-instrument dataset report (*NEW*)



Report for m84031_241114_020536_s4

PacBio

Dataset details

Name: Acq1_HG002-Cell1 (all samples)
Path: /collections/appslabvast/r84031/r84031_20241114_015813/1_A01/pb_formats/m84031_241114_020536_s4.hifi_reads.consensusreadset.xml
Unique ID: 0636d059-3423-4039-8e89-d6bd41926404
Created at: 2024-11-15T14:29:29.137Z
HiFi sequences: 6
HiFi bases: 105,301
Bio sample name: [multiple]
Well sample name: Acq1_HG002
Run name: 20241113_KuduVal_84031_Tray1
Movie name: m84031_241114_020536_s4
Instrument name: 84031
ICS version: 13.3.0.253824
Number of child datasets: 3
Number of HiFi BAM files: 3

On-instrument dataset PDF report is similar to SMRT Link Data Management dataset details (exported PDF) report and contains useful QC information for a dataset:

- Run setup information
- Sequencing performance metrics (e.g., read length, *P1* loading, run QC plots)

Note: Data Management dataset details report includes additional information about secondary analysis parameters and results that are not included in the on-instrument dataset report[2]

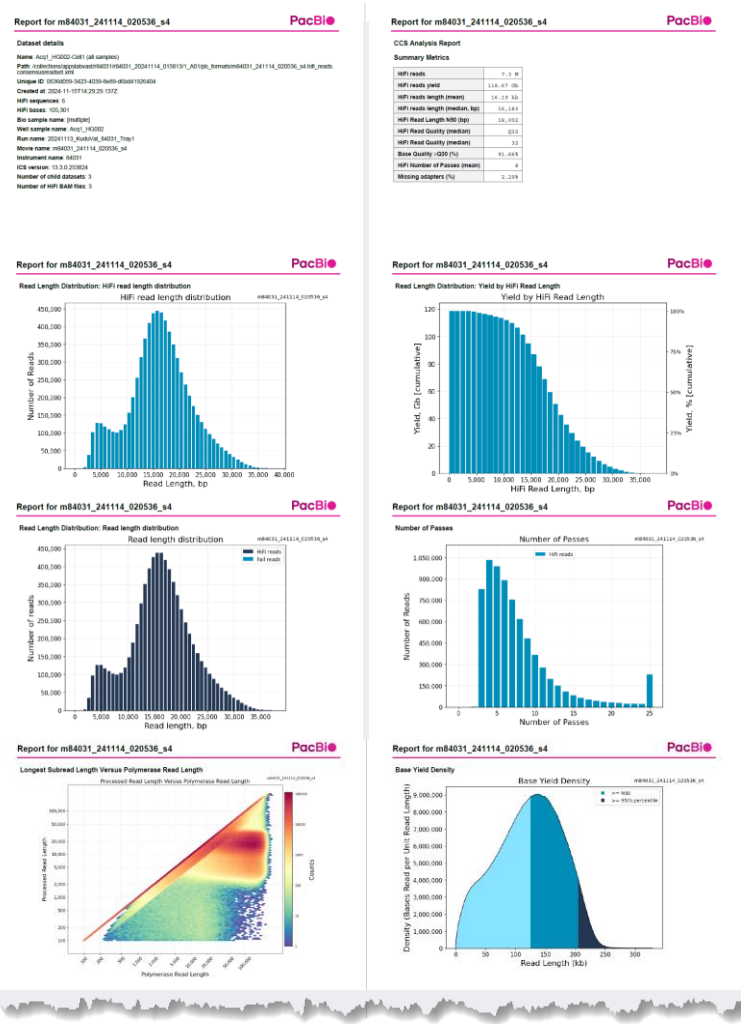
[1] Refer to PacBio BAM format specification page (https://pacbiofileformats.readthedocs.io/en/13.0/BAM.html) for details about PacBio output data file structures.

[2] **Note:** On-instrument dataset report is generated after CCS, methylation calling and demultiplexing are completed for a sample and **does not include** any secondary analysis parameters or results that may have been generated through auto-analysis in Run Design or through manual analysis by users.

Revio system v13.3 on-instrument dataset report

Example on-instrument dataset details report vs. SMRT Link Data Management dataset details report

Revio v13.3 on-instrument dataset details report



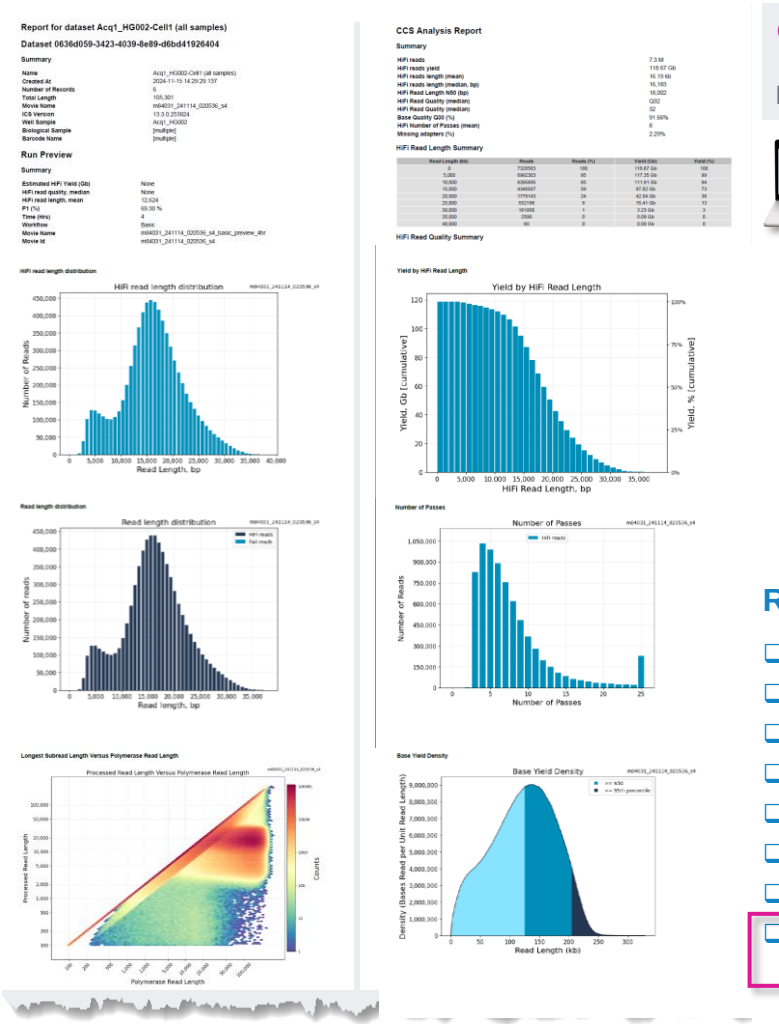
On-instrument dataset report (PDF) is auto-generated and saved in statistics folder[1]

```
r84001_20221122_134701
└─1_A01
  ├─fail_reads
  │ ├─m84001_250101_123456_s1.fail_reads.bam
  │ └─m84001_250101_123456_s1.fail_reads.bam.pbi
  ├─hifi_reads
  │ ├─m84001_250101_123456_s1.hifi_reads.bam
  │ └─m84001_250101_123456_s1.hifi_reads.bam.pbi
  ├─metadata
  ├─pb_formats
  ├─statistics
  │ └─m84001_250101_123456_s1.report.pdf
```

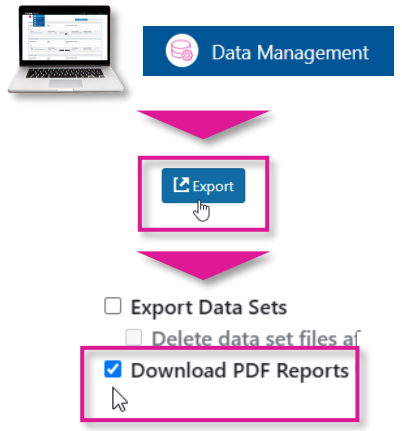
Report contents

- ❑ Dataset overview (metadata)
- ❑ CCS analysis report
- ❑ Methylation
- ❑ Demultiplexing report
- ❑ Loading report
- ❑ Raw data report
- ❑ Control report

SMRT Link Data Management dataset details report



Off-instrument dataset report is optionally generated in SMRT Link Data Management by user



Report contents

- ❑ Dataset overview (metadata)
- ❑ CCS analysis report
- ❑ Methylation
- ❑ Demultiplexing report
- ❑ Loading report
- ❑ Raw data report
- ❑ Control report
- ❑ Secondary analysis parameters and results

[1] **Note:** Copy of Revio v13.3 on-instrument dataset details PDF report can also be downloaded from **SMRT Link Data Management > Data > File Downloads page.**

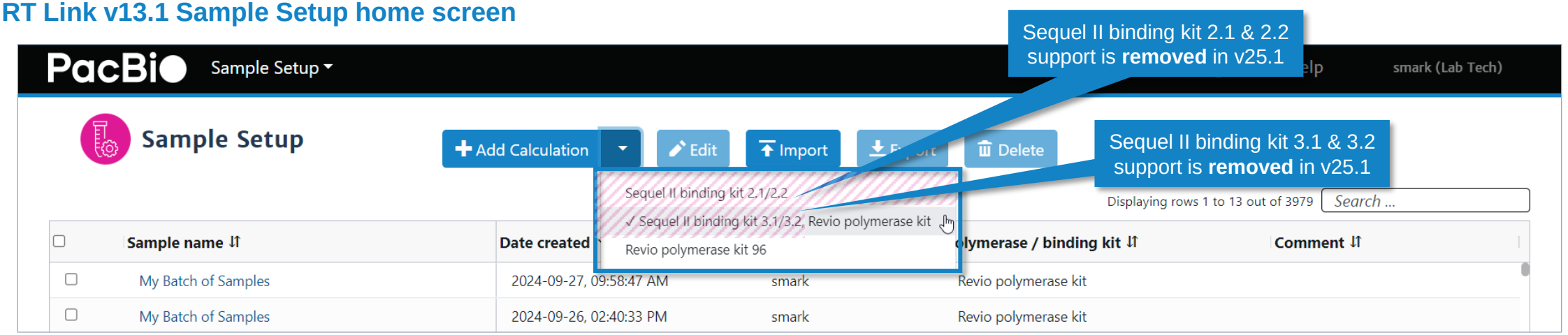
PacBio

Revio system v13.3
user experience improvements

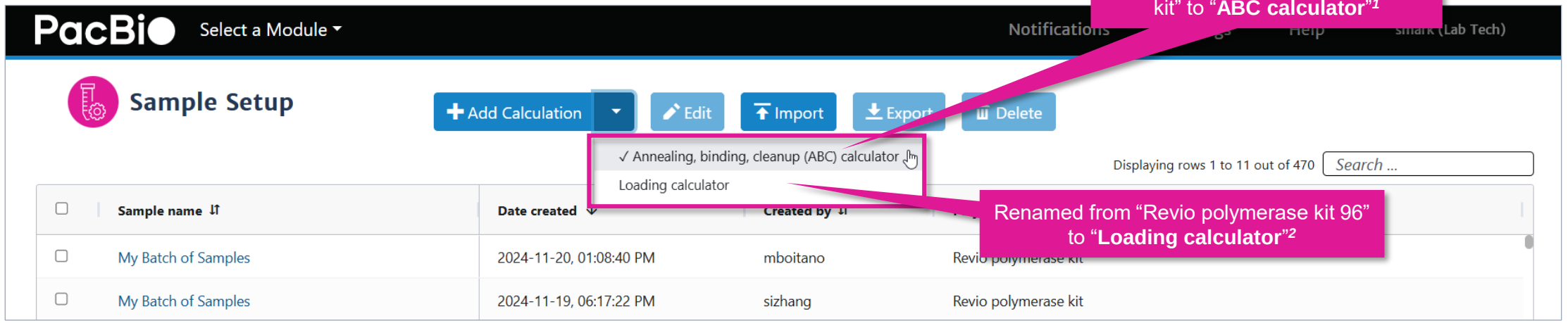
SMRT Link v25.1 Sample Setup user interface updates – Home screen

Updated Sample Setup home screen improves clarity of supported calculator features

SMRT Link v13.1 Sample Setup home screen



SMRT Link v25.1 Sample Setup home screen



[1] **Note:** To perform annealing, binding and cleanup with Revio SPRQ libraries, follow ABC instructions specified in the appropriate Procedure & checklist documentation.

[2] Loading calculator should be used to perform DNA sequencing control dilution and final sample dilution steps for Revio SPRQ libraries.

SMRT Link v25.1 Sample Setup user interface updates – General best practices

General best practices section includes additional clarifying instructions for preparing sequencing plate for loading[1]

General best practices

Eppendorf Lo-bind tubes (Eppendorf 022431021) are recommended for this protocol, but PCR tube strips (USA Scientific TempAssure 1402-4708) or 0.2 mL 96-well PCR plates are also acceptable. No difference in performance is expected across Lo-bind tubes, PCR tube strips, or plates.

To pipette-mix, gently pipette up and down until the solution appears homogenous, typically 10 times.

To prepare for the steps below, thaw the **Loading buffer** at **room temperature** and equilibrate the **SMRTbell cleanup beads** to **room temperature**. Store all other reagents on ice unless otherwise noted.

Mix reagent buffers with a brief vortex prior to use. Do not vortex enzymes.

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

To prepare the sequencing plate for loading: 1) Thaw in a **room temperature** water bath for **60 minutes**, protected from light; 2) Visually inspect the plate for any remaining frozen reagents by lifting the plate to eye-level, without inverting, and examining from all angles. If any reagents remain frozen, thaw for an additional **15 minutes**, protected from light; 3) Once thawed, vortex/shake to mix for **1 minute** at **1200 rpm** ; 4) Spin for **1 min** at **150 rcf** to ensure reagents are in the bottoms of each well; 5) Wipe with a new KimWipe to remove any moisture or contaminants from the plate foil.

To pipette accurately into the sequencing plate, dispense the sample against the middle of the well side wall and avoid immersing the pipette tip in the sample during blow out.

Thawing instructions are also found in ***Revio operations guide*** (102-962-600) and remain the **same** for both Revio sequencing plate and Revio SPRQ sequencing plate[2,3]

[1] Note: Following statement is **removed** from Sample Setup v25.1 General best practices section of ABC calculator: "If multiplexing, it is recommended to pool SMRTbell libraries prior to Sample Setup. If pooling after Sample Setup, pool samples in equimolar ratios. Only pool samples that were bound with the same sequencing polymerase."

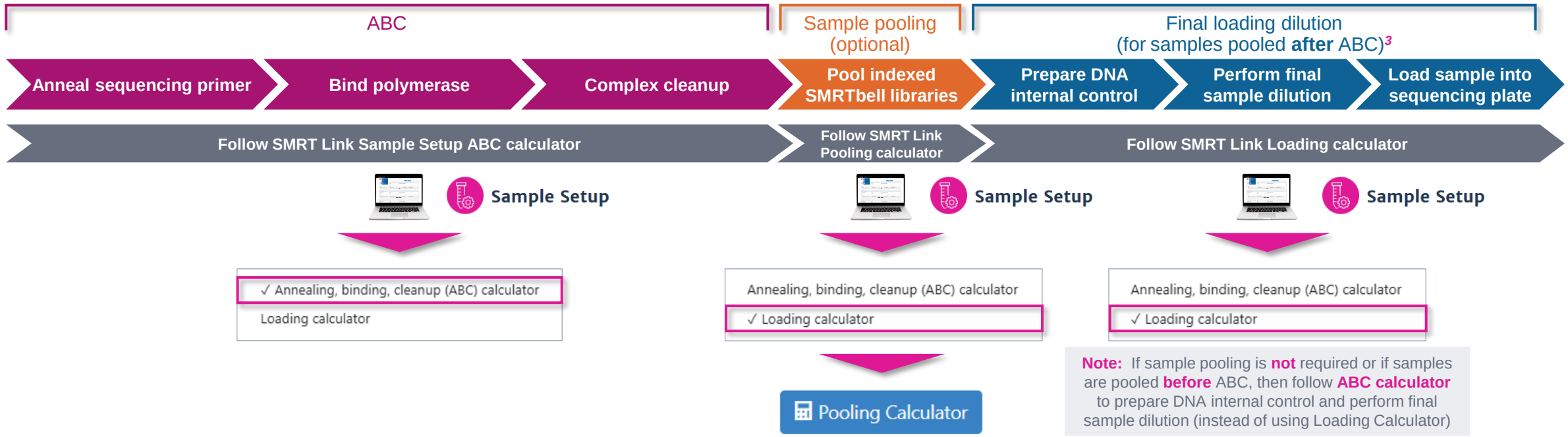
[2] If using the Loading calculator with Revio SPRQ libraries, follow the same instructions above to prepare the Revio SPRQ sequencing plate for loading.

[3] If reusing a Revio sequencing plate or Revio SPRQ sequencing plate: Store plate at 4°C protected from light – do not re-freeze. Sequencing plate can be stored for up to two weeks after first use.

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

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

Library type[1]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 **HiFi plex prep kit 96** **Kinnex single-cell RNA** **Kinnex full-length RNA** **Kinnex 16S rRNA**	**Revio polymerase kit** (102-817-600)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow SMRT Link v25.1 Sample Setup ABC calculator
		Sample pooling[2] (optional)	❑ Follow SMRT Link v25.1 Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link v25.1 Sample Setup Loading calculator (if pooling samples **after** ABC)[3]



[1] For binding **PureTarget libraries** with Revio polymerase kit, follow PureTarget library prep Procedure & checklist (103-329-400) instructions for ABC, pooling & final dilution steps. For binding **HiFi prep kit 96 WGS libraries** with Revio polymerase kit 96, follow HiFi prep kit 96 WGS library prep Procedure & checklist (103-420-700) for sample setup instructions.

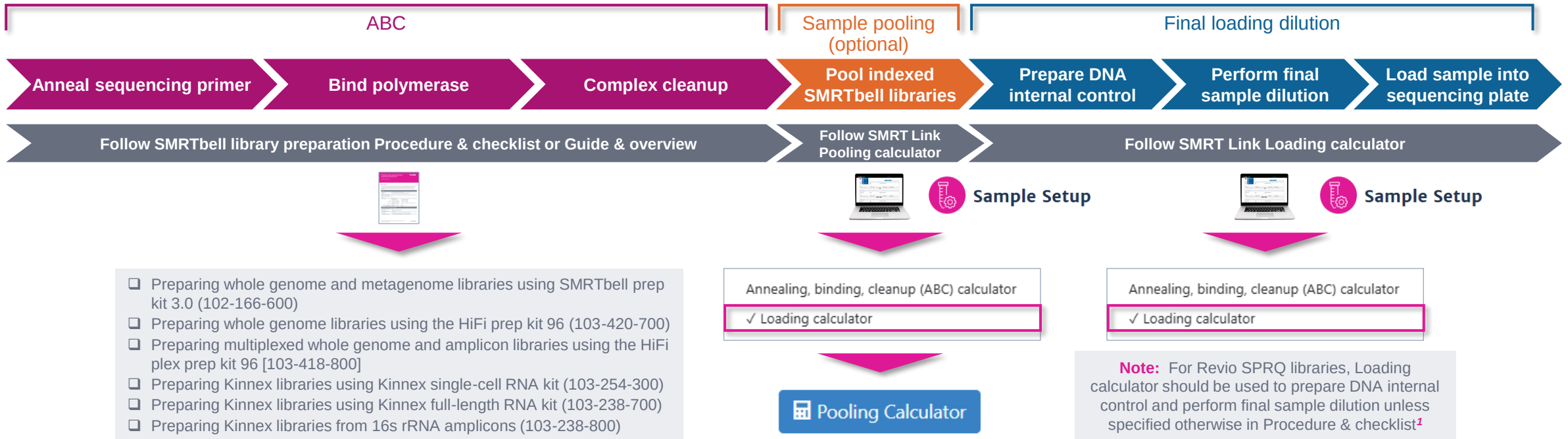
[2] For low-multiplexing applications, it is generally recommended to pool adapter-indexed HiFi libraries post-ABC to prevent any potential inhibitor in one sample from affecting the polymerase binding of all samples in a pool. **Note:** For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled prior to ABC step.

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

Sample setup workflow overview for Revio SPRQ polymerase libraries

For binding libraries with Revio SPRQ polymerase kit / Revio SPRQ polymerase kit 96, follow library prep Procedure & checklist instructions for annealing/binding/complex cleanup steps

Library type[1,2]	Polymerase kit	Sample setup workflow & procedural reference	
SMRTbell prep kit 3.0 **HiFi prep kit 96** **HiFi plex prep kit 96** **Kinnex single-cell RNA** **Kinnex full-length RNA** **Kinnex 16S rRNA**	**Revio SPRQ polymerase kit** (103-520-100) *or* **Revio SPRQ polymerase kit 96** (for HPK 96 libraries)	Anneal sequencing primer, bind polymerase, complex cleanup (ABC)	❑ Follow library prep Procedure & checklist
		Sample pooling[3] (optional)	❑ Follow SMRT Link v25.1 Sample Setup Pooling calculator
		Final loading dilution procedure	❑ Follow SMRT Link v25.1 Sample Setup Loading calculator



[1] For binding **PureTarget libraries** with Revio SPRQ polymerase kit, follow PureTarget library prep Procedure & checklist (103-329-400) instructions for sample setup ABC, pooling and final dilution steps.
[2] If preparing **HiFi prep kit 96 or HiFi plex prep kit libraries using automation**, refer to the appropriate automation Guide & overview documentation for sample setup instructions.
[3] For low-multiplexing applications, it is generally recommended to pool adapter-indexed HiFi libraries post-ABC to prevent any potential inhibitor in one sample from affecting the polymerase binding of all samples in a pool. **Note:** For high-multiplexing applications (e.g., ≥24-plex) using HiFi plex prep kit 96 (or other high-throughput kits), adapter-indexed HiFi libraries will typically be pooled prior to ABC step.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

Use SMRT Link or refer to Procedure & checklist documentation to prepare libraries for sequencing

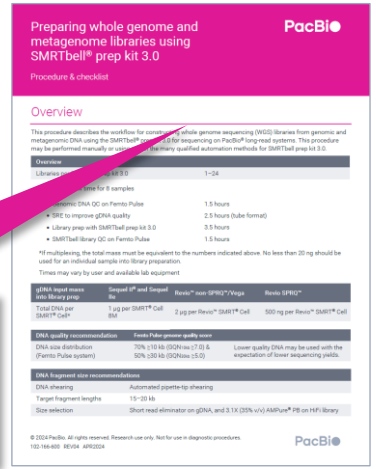
Revio polymerase kit

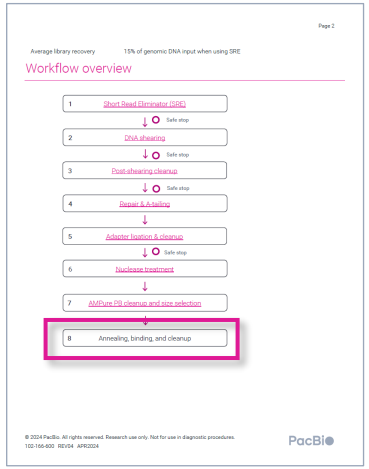
Revio SPRQ polymerase kit

SMRT Link v25.1

For binding SMRTbell libraries with Revio (non-SPRQ) polymerase kit, follow **SMRT Link Sample Setup ABC calculator** instructions

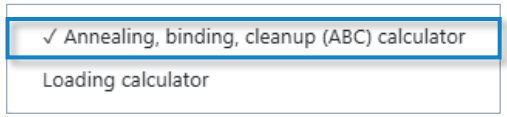
For binding SMRTbell libraries with Revio SPRQ polymerase kit, **follow ABC instructions described in application-specific Procedure & checklist**





Procedure & checklist

Sample Setup



No changes to on-plate loading concentration recommendations in SMRT Link v25.1 (compared to v13.1)

8. Annealing, binding, and cleanup (ABC)

This step is for preparing the SMRTbell library (25 µl) for sequencing on PacBio sequencers. The sequencing polymerase is stable once bound to the HiFi library and can be stored at 4°C for 1 month or at -20°C for at least 6 months. **If samples are pooled prior to ABC or a custom volume is required, see Appendix section A3.**

✔	Step	Instructions
8.1		Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔ Tube	Component	Volume
Light blue	Annealing buffer	12.5 µL
Light green	Standard sequencing primer	12.5 µL
	Total volume	**25 µL**

On-plate loading concentration recommendations are **included** in protocol (**same as in SMRT Link**)[1]

[1] **Note:** Recommended OPLC conditions are the **same** for both Revio non-SPRQ chemistry and Revio SPRQ chemistry.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

Entering sample information for annealing, binding and cleanup procedure

Revio polymerase kit

SMRT Link v25.1 Sample Setup ABC calculator **does not support Revio SPRQ polymerase kit**

< Sample group >

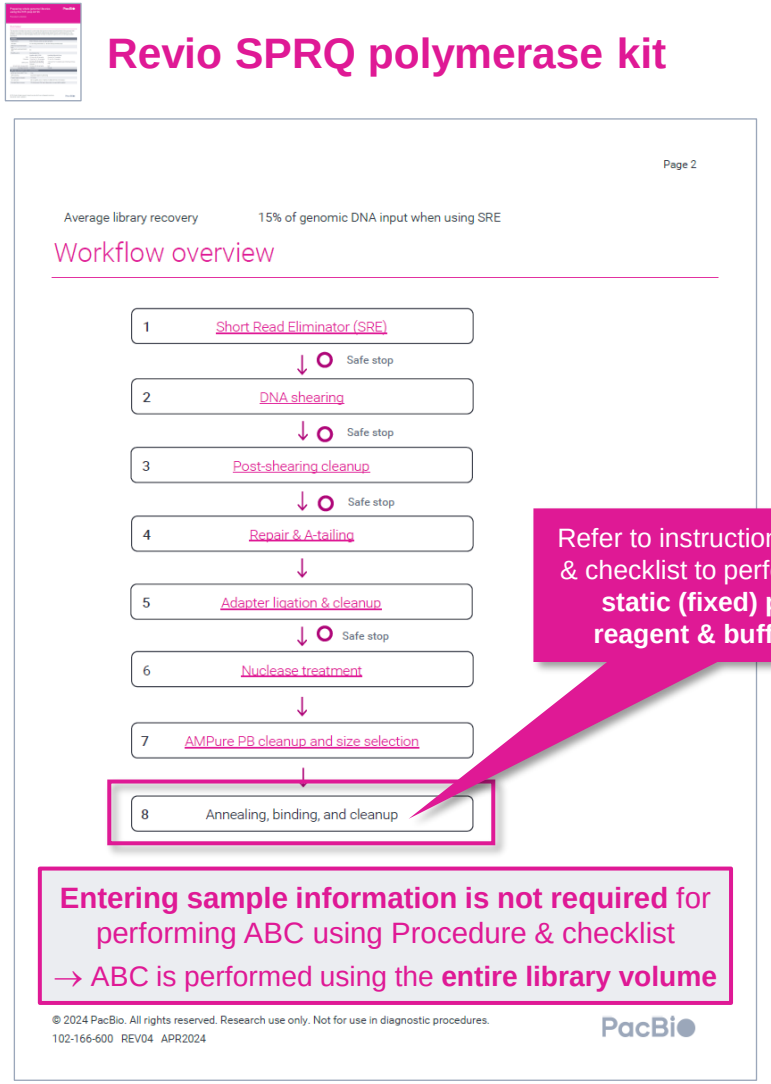
Copy | Remove | Lock | Download CSV

Name	
Application	Human WGS
Library type	Standard
Polymerase kit	Revio polymerase kit
Number of samples*	samples
SMRT Cells per sample*	cells
Available volume per sample*	uL
Insert size*	bp
Sample concentration*	ng/uL
Cleanup anticipated yield	75 %
Concentration on plate*	pM Recommended: 200-300 pM
Minimum pipetting volume	1 uL
Comment	

Enter all required sample information into SMRT Link ABC calculator form[1]

→ ABC is performed using **only the required volume of library**

Revio SPRQ polymerase kit



Refer to instructions in Procedure & checklist to perform ABC using **static (fixed) per-sample reagent & buffer volumes**

Entering sample information is not required for performing ABC using Procedure & checklist

→ ABC is performed using the **entire library volume**

[1] ⚠ Note: SMRT Link is not certified as HIPAA-compliant; please do not incorporate any private or confidential information in sample name field

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

Entering sample information for annealing, binding and cleanup procedure

Revio polymerase kit

Prior to ABC, measure SMRTbell library DNA concentration after final AMPure PB bead cleanup and size selection step in Procedure & checklist

7 AMPure PB cleanup and size selection

Sample Setup

	< Sample group >	< Sample group >
	Copy Remove Lock Download CSV	Copy Remove Lock Download CSV
Name	Sample 1	Sample 2
Application	Human WGS	Human WGS
Library type	Standard	Standard
Polymerase kit	Revio polymerase kit	Revio polymerase kit
Number of samples	1 samples	
Cells per sample	1 cells	
e per sample	25 uL	25 uL
	17362 bp	16934 bp
Sample concentration	40 ng/uL	30 ng/uL
Cleanup anticipated yield	75 %	75 %
Concentration on plate	225 pM Recommended: 200-300 pM	225 pM Recommended: 200-300 pM
Minimum pipetting volume	1 uL	1 uL

DNA concentration must be **less than 60 ng/µL** to proceed to ABC following **SMRT Link calculator** instructions[1]

Note: After AMPure PB bead size selection, default SPK 3.0 library elution volume = **26 µL**

Revio SPRQ polymerase kit

Prior to ABC, measure SMRTbell library DNA concentration after final AMPure PB bead cleanup and size selection step in Procedure & checklist

7 AMPure PB cleanup and size selection

8 Annealing, binding, and cleanup

✔	Step	Instructions for AMPure PB bead size selection
	7.1	Make a 35% v/v dilution of AMPure PB beads by adding 1.75 mL of resuspended AMPure PB beads to 3.25 mL of elution buffer. The 35% dilution can be stored at 4°C for 30 days. **Note:** The AMPure PB dilution may be scaled as appropriate for smaller/larger scale projects.
	7.2	Add 3.1X v/v (155 µL) of resuspended, room the previous step.
	7.10	Remove samples from the magnet and **immediately** add **26 µL** of **elution buffer** to each sample.
	7.11	Pipette-mix the beads until evenly distributed.
	7.15	Take a **1 µL** aliquot from each tube and dilute with **9 µL** of **elution**. Measure the DNA concentration with a Qubit fluorometer using the 1x dsDNA H. ate the total mass. The final overall recovery should be 10–30% as measured from gD mass to completed SMRTbell library (includes SRE, shearing, library prep, and ABC). **Recommended**: Further dilute each aliquot to 250 **µL** with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.
		DNA concentration must be less than 60 ng/µl to proceed to ABC. Failure to dilute DNA below 60 ng/µl will result in low P1 loading.
	7.16	Proceed to step 8 to prepare library for sequencing on Vega or Revio SPRQ. OR Proceed to SMRT Link Sample Setup for preparing samples for Revio non-SPRQ chemistry or Sequel II/e.

SAFE STOPPING POINT - Store at 4°C <1 month and -20°C for long term

Note: After AMPure PB bead size selection, default SPK 3.0 library elution volume = **26 µL**

DNA concentration must be **less than 60 ng/µL** to proceed to ABC following **Procedure & checklist** instructions

[1] Example **sample concentration** and other sample information values shown in SMRT Link ABC calculator form are for illustrative purposes. For WGS samples processed with SMRTbell prep kit 3.0, typical library construction yields are typically in the range of ~13% – 34%.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

1. Annealing sequencing primer

Revio polymerase kit

✓	Step	Instructions
	1.1	For each sample in each batch, add the following components in the order and volume listed to a new Lo-bind tube, one per sample.

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
		Sample (Pooled SMRTbells)	8.5 uL	10.5 uL
	light blue	Annealing buffer	4.3 uL	5.3 uL
	light green	Standard sequencing primer	4.3 uL	5.3 uL
		Total volume	**17.0 uL**	**21.0 uL**

1.2	Pipette-mix.	
1.3	Incubate at **room temperature** for **15 minutes.**	
1.4	Proceed to the next step of the protocol.	

For each sample, add the **calculated volumes** of annealing buffer + sequencing primer shown in table **directly** to the sample

Revio SPRQ polymerase kit

✓	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✓	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	**25 µL**

Prepare **master mix** containing required **(fixed) per-reaction volumes** of annealing buffer + sequencing primer and then distribute to each sample

8.2	Pipette-mix the **Annealing mix** and quick spin to collect liquid.
8.3	Add **25 µL** of the **Annealing mix** to each library. Total volume should equal **50 µL.**
8.4	Pipette-mix each sample and quick spin to collect liquid.
8.5	Incubate at room temperature for **15 minutes**
8.6	During primer incubation, prepare the polyme

Add **fixed volume (25 µL)** of Annealing mix to each sample. Total primer annealing reaction volume for each sample is **fixed at 50 µL**

Use the **fixed per-reaction volumes** of annealing buffer & sequencing primer shown in table and **do not adjust reagent volumes** based on measured DNA library conc. or insert size

Appendix

If samples are pooled prior to ABC or if a custom volume is required, use the calculations below to determine reagent volumes based on input sample volume:[1]

	SMRTbell library	Annealing buffer	Standard sequencing primer	Polymerase dilution
Volume (µl)	x	x/2	x/2	x*2
Example	100	50	50	200

[1] For example, if your starting library volume is 100 µL instead of 25 µL, then you simply use 4 times as much Annealing buffer and sequencing primer to make 100 µL of the master Annealing mix, and then add the 100 µL of master Annealing mix to your 100 µL of starting library to produce a total annealing reaction volume of 200 µL instead of 50 µL.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

2. Binding sequencing polymerase

Revio polymerase kit

✓	Step	Instructions
		Dilute Sequencing polymerase
	2.1	Add the following components in the order and volume listed to a new Lo-bind tube. The volume of reaction mix is sufficient for all samples in all batches.

✓	Tube	Component	Reaction Mix 2 (RM2)
	purple	Sequencing polymerase	2.5 uL
	yellow	Polymerase buffer	39.2 uL
		Total volume	**41.7 uL**

2.2	Pipette-mix **RM2**.
	RM2 must be used immediately. Discard any remainder.
	Bind Sequencing polymerase
2.3	Add **RM2** in the specified volume to each sample in each bat

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
		Sample from step 1	17.0 uL	21.0 uL
		RM2	17.0 uL	21.0 uL
		Total volume	**34.0 uL**	**42.0 uL**

2.4	Pipette-mix each sample.
2.5	Incubate at **room temperature** for **15 minutes**.
2.6	Proceed to the next step of the protocol.
	SAFE STOPPING POINT – Store at 4°C for up to 4 weeks.

Prepare **polymerase dilution master mix** containing **calculated** volumes of sequencing polymerase + polymerase buffer

Add **calculated volume** of polymerase dilution master mix to each sample. Polymerase binding reaction volume will **vary** depending on specific sample & reagent volumes used.

Revio SPRQ polymerase kit

✓	Step	Instructions
	8.7	To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase Dilution

✓	Tube	Component	Volume
	Yellow	Polymerase buffer	47 µL
	Purple	Sequencing polymerase	3 µL
		Total volume	**50 µL**

8.8	Pipette mix the **polymerase dilution** and quick-spin to collect liquid.
8.9	Add **50 µL of polymerase dilution** to primer annealed sample. Total volume should equal **100 µL**.
8.10	Pipette-mix each sample and quick-spin to collect liquid.
8.11	Incubate at **room temperature for 15 minutes.**
8.12	Proceed immediately to the next step of the protocol

Prepare **polymerase dilution master mix** containing **fixed per-reaction volumes** of sequencing polymerase + polymerase buffer

Add **fixed volume (50 µL)** of polymerase dilution master mix to each sample. Polymerase binding reaction volume is fixed at **100 µL**

Appendix

If samples are pooled prior to ABC or if a custom volume is required, use the calculations below to determine reagent volumes based on input sample volume:[1]

	SMRTbell library	Annealing buffer	Standard sequencing primer	Polymerase dilution
Volume (µl)	x	x/2	x/2	x*2
Example	100	50	50	200

[1] For example, if your starting library volume is 100 µL instead of 25 µL, then you simply prepare 4 times as much polymerase dilution master mix, which would be 200 µL instead of 50 µL, and then add the 200 µL of polymerase dilution to your 200 µL of annealed library to produce a total binding reaction volume of 400 µL instead of 100 µL.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

3. Purification of polymerase-bound SMRTbell complexes

Revio polymerase kit

✓	Step	Instructions

Add the specified volume of **Dilution buffer** to each sample in each batch.

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
		Sample from step 2	34.0 uL	42.0 uL
	blue	Dilution buffer	66.0 uL	58.0 uL
		Total volume	100.0 uL	100.0 uL

Bring each sample to **100 µL** total volume with Dilution buffer

3.2 Pipette-mix each sample.

Add the specified volume of **SMRTbell cleanup beads** to each sample in each batch.

3.3

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
		SMRTbell cleanup beads	120.0 uL	120.0 uL

Perform **1.2X bead cleanup** by adding **fixed** volume (**120 µL**) of SMRTbell cleanup beads to each diluted sample

...distributed. Quick-spin to collect contents.

...utes.

...ack until the beads separate fully from the solution.

3.7 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant. Do **not** wash the beads with ethanol.

Immediately add the specified volume of room temperature **Loading buffer** to each sample in each batch.

3.8

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
	green	Loading buffer	50.0 uL	50.0 uL

3.9 Pipette-mix each sample. Quick-spin to collect contents.

3.10 Incubate at **room temperature** for **5 minutes** to ...elute sample.

3.11 Place each tube in a magnetic separation rack until the beads separa...

...urbing the be...

Elute each cleaned complex in **fixed** volume (**50 µL**) **of Revio polymerase loading buffer** by incubating for **5 min** at RT

Optionally perform DNA concentration measurement using Qubit assay before proceeding to sequencing control dilution step

3.14 Optionally, quantify samples using a Qubit instrument by taking a 1 uL aliquot from each sample.

Revio SPRQ polymerase kit

✓	Step	Instructions

Post-binding cleanup with 1X SMRTbell cleanup beads

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

Perform **1.0X bead cleanup** by adding **fixed** volume (**100 µL**) of SMRTbell cleanup beads directly to each sample (**100 µL**)

8.14 Pipette-mix the beads until evenly distributed and qu...in if necessary to collect all liquid from the sides of the tube.

8.15 Incubate at **room temperature** for **10 minutes** t...

8.16 Place sample on an appropriate magnet and all...

8.17 Slowly remove the cleared supernatant without ... **DO NOT USE EtOH.** Proceed immediately to the elution. It is important not to let the beads dry out.

8.18 Remove sample from the magnet and **immediately** add... resuspend the beads by pipette mixing.

	Revio SPRQ	Vega
Loading buffer	25 µL	50 µL

Elute each cleaned complex in fixed volume (**25 µL**) **of Revio SPRQ polymerase loading buffer**[1] by incubating for **15 min** at RT

8.19 Quick-spin the samples to collect any liquid from the sides of the tube.

8.20 Incubate at **room temperature for 15 minutes to** elute DNA

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

8.22 Slowly remove the cleared eluate without disturbi... Discard the old tube with beads

Perform DNA concentration measurement using Qubit assay (**required**)

8.23 Use **1 µL** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
Important: The **Qubit Flex** instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96. Concentration readings will not be accurate.

8.24 Proceed to the **Loading Calculator** in SMRT Link v25.1or higher to calculate the final dilution for adding the sample to Sequencing reagent plate. The recommended loading concentration is 200 – 300 pM.

Polymerase-bound libraries can be store... sequencing. Polymerase-bound libraries... sequencing performance.

Proceed to SMRT Link Loading Calculator to perform sequencing control dilution step and final loading dilution step using recommended OPLC

PROTOCO...

[1] Slowly pipette Revio SPRQ polymerase loading buffer as it is a viscous solution.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

4. Sequencing control dilution

Revio polymerase kit

Perform three sequential dilution steps of **Sequencing control** using **Dilution buffer**. Use a new Lo-bind tube for each dilution step. The volume is sufficient for all samples in all batches.

✓	Step	Instructions

Perform a first dilution by adding **Dilution buffer** to **Sequencing control**.

✓	Tube	Component	Dilution 1
4.1	blue	Dilution buffer	19.0 uL
	red	Sequencing control	1.0 uL
		Total volume	**20.0 uL**

4.2 Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.

Perform a second dilution by adding **Dilution buffer** to **Dilution 1.**

✓	Tube	Component	Dilution 2
4.3	blue	Dilution buffer	19.0 uL
		Dilution 1	1.0 uL
		Total volume	**20.0 uL**

4.4 Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.

Perform a third dilution by adding **Dilution buffer** to **Dilution 2.**

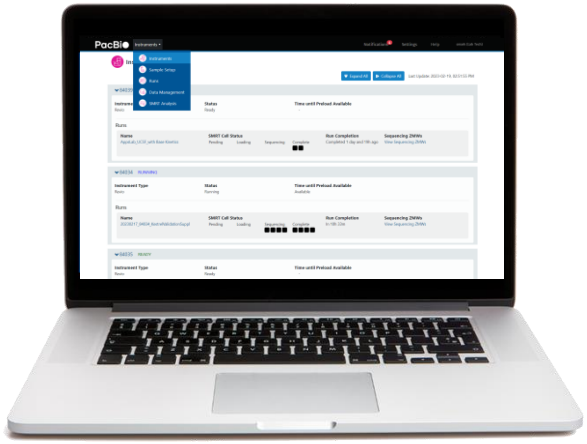
✓	Tube	Component	Dilution 3
4.5	blue	Dilution buffer	19.0 uL
		Dilution 2	1.0 uL
		Total volume	**20.0 uL**

4.6 Pipette-mix the dilution. Quick-spin to collect contents.

4.7 Discard Dilution 1 and Dilution 2.

4.8 Keep Dilution 3 on ice.

Revio SPRQ polymerase kit



SMRT Link v25.1

Sample Setup

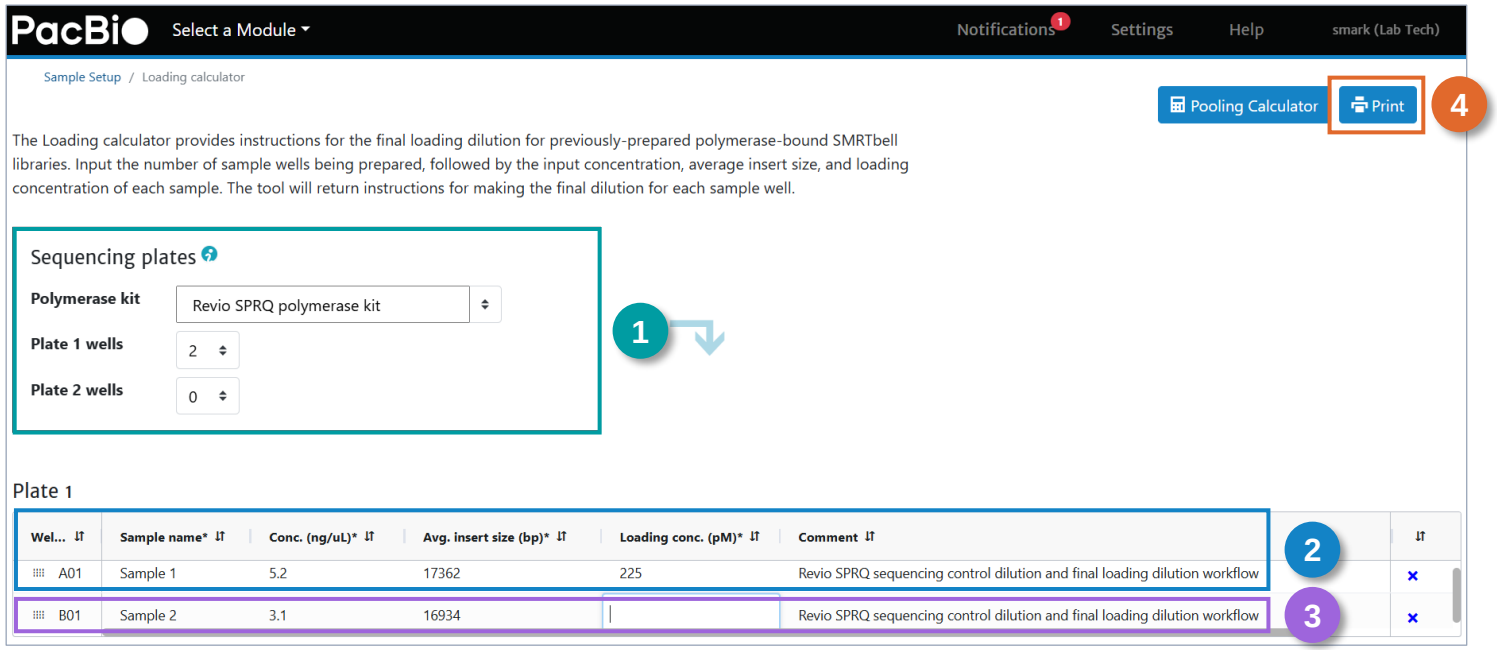
✓ Annealing, binding, cleanup (ABC) calculator

Loading calculator

SMRT Link Loading Calculator procedure for Revio SPRQ polymerase libraries

Enter required sample information to perform sequencing control dilution and final loading dilution steps

1. **Specify polymerase kit and number of samples to use**
   - Specify polymerase kit type
   - Specify the number (1-4) of sample wells to use per sequencing plate
   - **Note:** If you are using only one sequencing plate, specify 0 for Plate 2
2. **Enter information for first sample well**
   - Sample name
   - Concentration (ng/μL)
   - Average insert size (in base pairs)
   - Loading concentration (in pM)
   - Comments (optional)
   - **Note:** If using a partially-used sequencing plate, can delete a Well ID by clicking on the '**x**' button at right-hand side of table
3. **Repeat Step 2 for additional sample wells**
   - **Note:** All sample wells must be filled in for the instructions to display.
4. **Print instructions (optional)**
   - To print the calculation(s) and instructions, click the Print button.



Loading Calculator outputs instructions for sequencing control dilution and final loading dilution procedure

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

4. Sequencing control dilution (cont.)

Revio polymerase kit

Perform three sequential dilution steps of **Sequencing control** using **Dilution buffer**. Use a new Lo-bind tube for each dilution step. The volume is sufficient for all samples in all batches.

✓	Step	Instructions
	4.1	Perform a first dilution by adding **Dilution buffer** to **Sequencing control**.

✓	Tube	Component	Dilution 1
	blue	Dilution buffer	19.0 uL
	red	Sequencing control	1.0 uL
		Total volume	**20.0 uL**

	4.2	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.
	4.3	Perform a second dilution by adding **Dilution buffer** to **Dilution 1.**

✓	Tube	Component	Dilution 2
	blue	Dilution buffer	19.0 uL
		Dilution 1	1.0 uL
		Total volume	**20.0 uL**

	4.4	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.
	4.5	Perform a third dilution by adding **Dilution buffer** to **Dilution 2.**

✓	Tube	Component	Dilution 3
	blue	Dilution buffer	19.0 uL
		Dilution 2	1.0 uL
		Total volume	**20.0 uL**

	4.6	Pipette-mix the dilution. Quick-spin to collect contents.
	4.7	Discard Dilution 1 and Dilution 2.
	4.8	Keep Dilution 3 on ice.

Perform 3 sequential 1:20 serial dilutions using **Revio sequencing control** included in **Revio polymerase kit**[1]

Revio SPRQ polymerase kit

Perform three sequential dilution steps of **Sequencing control** using **Dilution buffer**. Use a new Lo-bind tube for each dilution step. The volume is sufficient for all samples in all batches.

✓	Step	Instructions
	1.1	Perform a first dilution by adding **Dilution buffer** to **Sequencing control**.

✓	Tube	Component	Dilution 1
	blue	Dilution buffer	19.0 uL
	red	Sequencing control	1.0 uL
		Total volume	**20.0 uL**

	1.2	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.
	1.3	Perform a second dilution by adding **Dilution buffer** to **Dilution 1.**

✓	Tube	Component	Dilution 2
	blue	Dilution buffer	19.0 uL
		Dilution 1	1.0 uL
		Total volume	**20.0 uL**

	1.4	Pipette-mix the dilution. Quick-spin to collect contents. Keep on ice.
	1.5	Perform a third dilution by adding **Dilution buffer** to **Dilution 2.**

✓	Tube	Component	Dilution 3
	blue	Dilution buffer	19.0 uL
		Dilution 2	1.0 uL
		Total volume	**20.0 uL**

	1.6	Pipette-mix the dilution. Quick-spin to collect contents.
	1.7	Discard Dilution 1 and Dilution 2.
	1.8	Keep Dilution 3 on ice.

Perform 3 sequential 1:20 serial dilutions using **Revio SPRQ sequencing control** included in **Revio SPRQ polymerase kit**[2]

1 Revio polymerase kit (102-817-600) includes Revio sequencing control PN 102-798-000.
2 Revio SPRQ polymerase kit (103-520-100) includes Revio SPRQ sequencing control PN 103-508-800.

Sample setup workflow details for Revio polymerase & Revio SPRQ polymerase

5. Final loading dilution

Revio polymerase kit

✓	Step	Instructions

For each sample in each batch, add the following components to each sample tube from Step 3.

✓	Tube	# of Samples in Batch	Sample 1 1	Sample 2 1
		Sample	50.0 uL	50.0 uL
		Diluted sequencing control (Dilution 3)	3.0 uL	3.0 uL
	green	Loading buffer	47.0 uL	47.0 uL
		Total volume	**100.0 uL**	**100.0 uL**

5.1	
5.2	Pipette-mix each sample.
5.3	Protect samples from light.
5.4	Discard any unused Dilution 3.
5.5	Optionally, quantify samples using
	SAFE STOPPING POINT – Store protected from light at 4°C for up to 24 hours.

Add **fixed** volumes of **sample (50 µL) + diluted sequencing control (3 µL) + Revio polymerase loading buffer (47 µL)** to bring final loading dilution volume to **100 µL (fixed)**

Revio SPRQ polymerase kit

✓	Step	Instructions

For each sample, add the following components in the order and volume listed to a new Lo-bind tube.

✓	Tube	Plate:Well	Sample 1 1 : A01	Sample 2 1 : B01
		Sample	12.7 uL	20.8 uL
		Diluted sequencing control (Dilution 3)	1.0 uL	1.0 uL
	green	Loading buffer	12.3 uL	4.2 uL
		Total volume	**26.0 uL**	**26.0 uL**

2.1	
2.2	Pipette-mix each sample.
2.3	Protect samples from light.
2.4	Discard any unused Dilution 3.
2.5	Optionally, quantify samples using a Qub
	SAFE STOPPING POINT – Store protected from light at 4°C for up to 24 hours.

Add **calculated required volume of sample + 1 µL diluted sequencing control (fixed) + calculated required volume of Revio SPRQ polymerase loading buffer**[1] to bring final loading dilution volume to **26 µL (fixed)**

6. Sample loading on sequencing plate

Revio polymerase kit

✓	Step	Instructions
	6.1	Load exactly **95 uL** of sample per well on a sequencing plate.
	6.2	Seal the sequencing plate with a Sample well seal.
	6.3	Spin the sequencing plate in a centrifuge at **150 rcf** for **30 seconds**.
	6.4	Inspect the sample wells to ensure there are no bubbles and the sample volume is at the bottom of the well. If bubbles are present, forcefully tap the plate on the benchtop to remove and **return to step 6.3.**
	6.5	Load the sequencing plate onto the sequencing instrument, avoiding any disruption to the sample.

Load **exactly 95 µL (fixed)** of final loading dilution onto **Revio sequencing plate**[2]

Revio SPRQ polymerase kit

✓	Step	Instructions
	3.1	Load exactly **23 uL** of sample per well on a sequencing plate.
	3.2	Seal the sequencing plate with a Sample well seal.
	3.3	Spin the sequencing plate in a centrifuge at **150 rcf** for **30 seconds**.
	3.4	Inspect the sample wells to ensure there are no bubbles and the sample volume is at the bottom of the well. If bubbles are present, forcefully tap the plate on the benchtop to remove and **return to step 3.3.**
	3.5	Load the sequencing plate onto the sequencing instrument, avoiding any disruption to the sample.

Load **exactly 23 µL (fixed)** of final loading dilution onto **Revio SPRQ sequencing plate**[2]

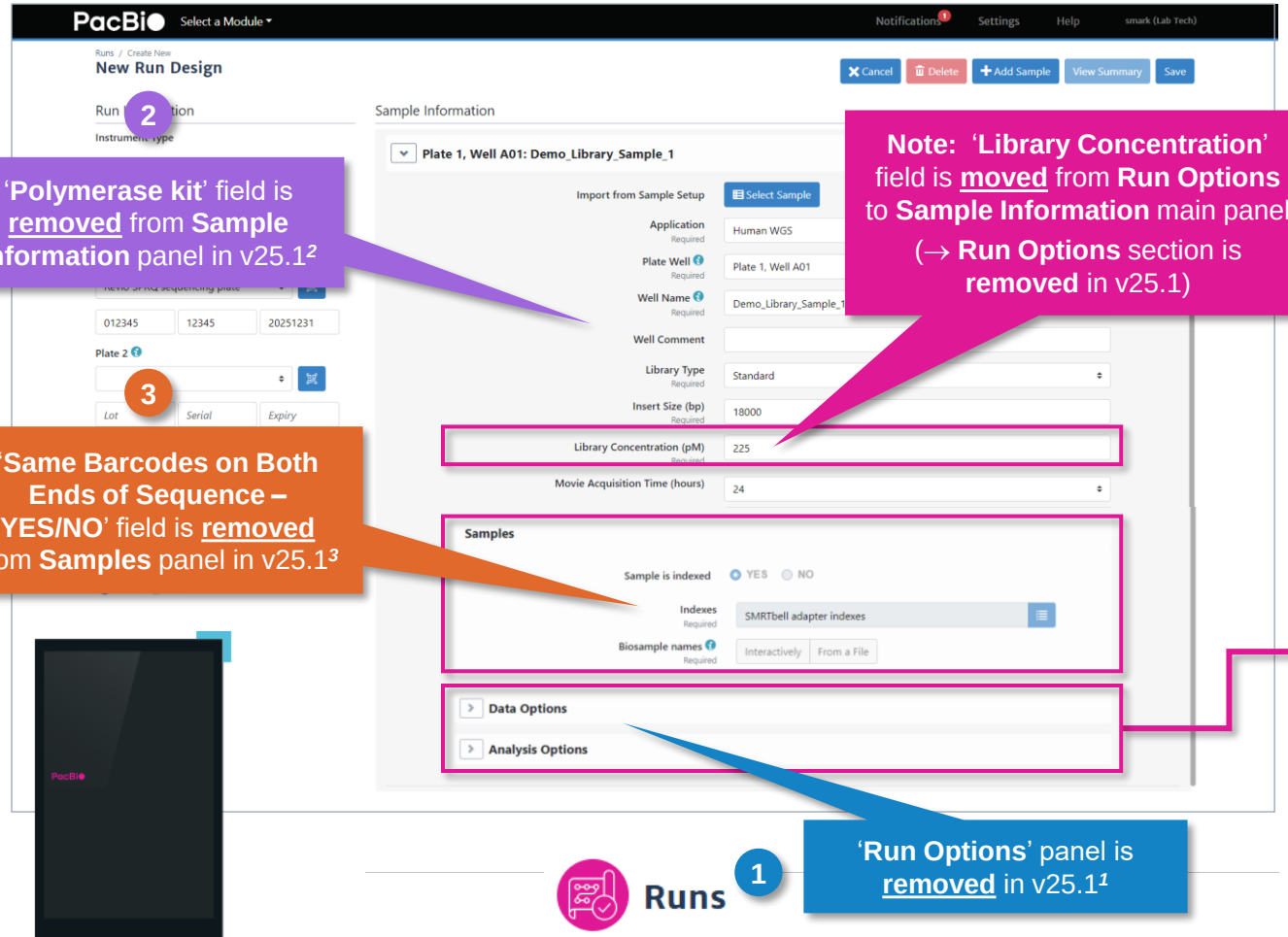
[1] Revio SPRQ polymerase loading buffer is a **viscous** solution and should be pipetted **slowly**.

[2] Load **exactly** the required volume of sample (95 µL for non-SPRQ chemistry or 23 µL for SPRQ chemistry) into the sequencing plate – Do not load less or more than the stated required volume as this can have a negative impact on sequencing performance.

SMRT Link v25.1 Run Design user interface updates

SMRT Link v25.1 Revio system Run Design page layout is streamlined to remove obsoleted/unsupported fields

Revio system Run Design v25.1 example – Human WGS application



'**Polymerase kit**' field is **removed** from **Sample Information** panel in v25.1[2]

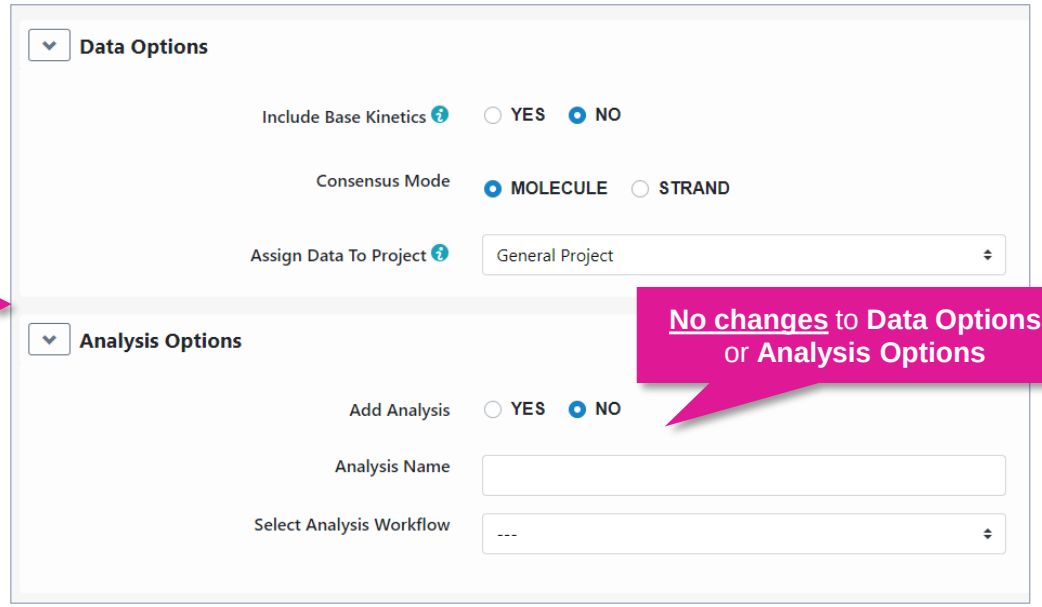
'**Same Barcodes on Both Ends of Sequence – YES/NO**' field is **removed** from **Samples** panel in v25.1[3]

Note: '**Library Concentration**' field is **moved** from **Run Options** to **Sample Information** main panel (→ **Run Options** section is **removed** in v25.1)

'**Run Options**' panel is **removed** in v25.1[1]

Revio system Run Design fields/options listed below are removed from SMRT Link v25.1

1 Run Options panel	Run Options
2 Polymerase kit	Polymerase Kit (Required)
3 Same Barcodes on Both Ends of Sequence	Same Barcodes on Both Ends of Sequence: YES / NO

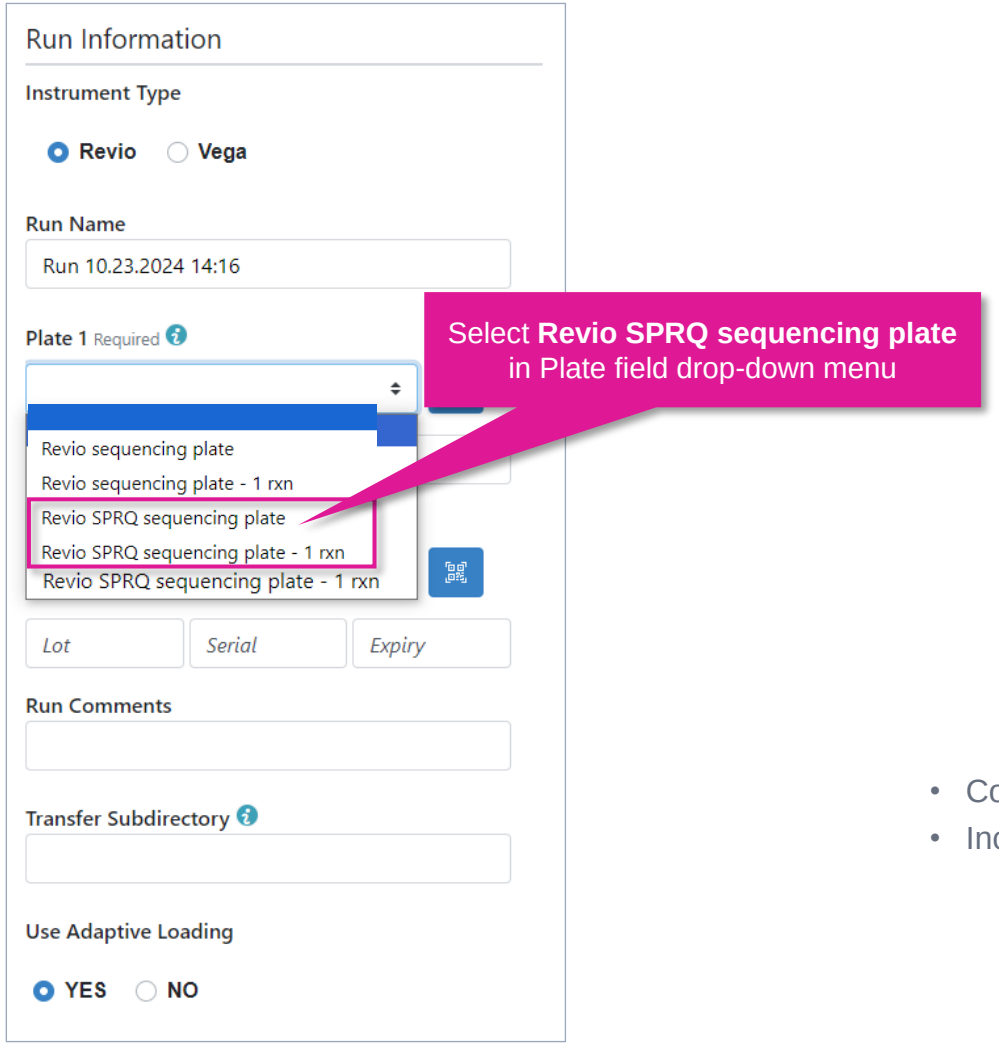


No changes to **Data Options** or **Analysis Options**

[1] **Note:** **'Run Options'** panel is removed since 'Library Concentration' field is moved (from Run Options) to Sample Information main panel.
[2] **Note:** **'Polymerase kit'** field is removed since all required information about the sequencing chemistry version is automatically provided by the sequencing plate information fields.
[3] **Note:** **'Same Barcodes on Both Ends of Sequence'** field is removed since Revio system **only** supports on-instrument demultiplexing of **symmetrically-indexed** samples. For demultiplexing of non-symmetrically indexed samples a demultiplexing analysis job can be performed off-instrument in SMRT Link.

SMRT Link v25.1 Run Design user interface updates – Run information panel

Updated Run Information panel supports new Revio SPRQ consumables



Select **Revio SPRQ sequencing plate** in Plate field drop-down menu

Runs

Revio SPRQ sequencing plate – 4 rxn (103-504-900)[1]

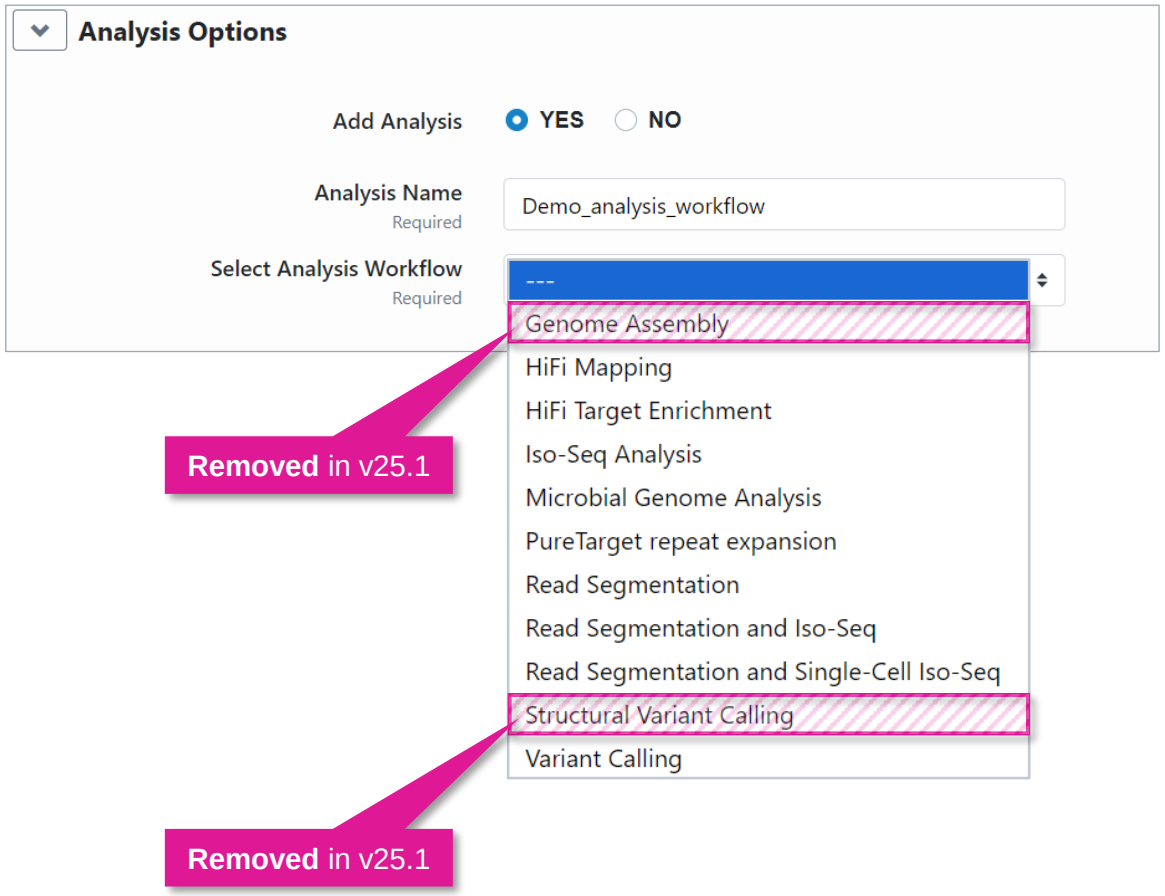
- Contains reagents for sequencing 4 Revio SMRT Cells on the Revio system
- Includes foil seals to prevent sample evaporation

[1] **Revio SPRQ sequencing plate – 1 rxn** (103-512-700) consumable is also available and contains reagents for sequencing 1 Revio SMRT Cell on the Revio system.

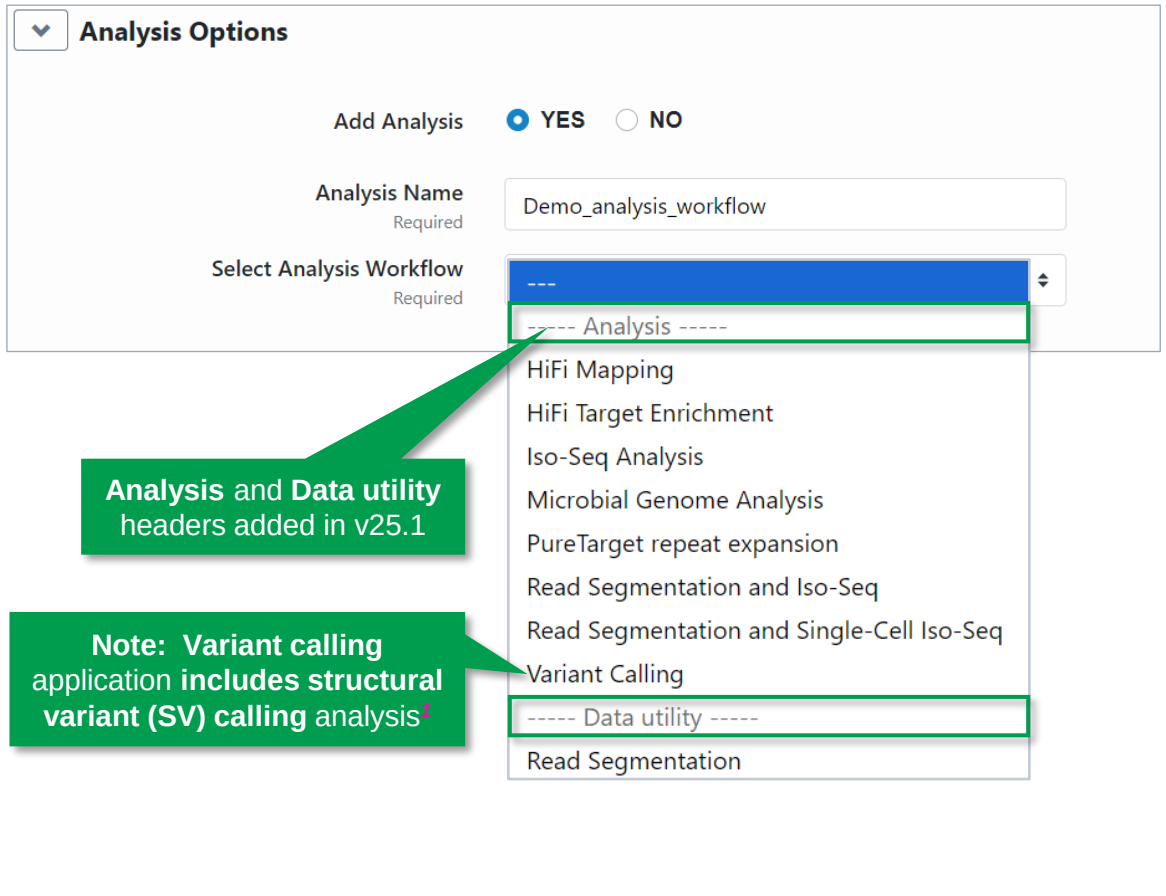
SMRT Link v25.1 Run Design user interface updates – Analysis Options

Updated Analysis Options section features a more streamlined analysis workflow dropdown menu

Runs **SMRT Link v13.1**



Runs **SMRT Link v25.1**



[1] SMRT Link v25.1 Variant Calling application now enables option to **skip** DeepVariant analysis – thus allowing only SV calling results to be output if desired.

SMRT Link v25.1 Run Details report updates – Run QC metrics table

Key primary sequencing QC metrics reported in Run Details table remain the same

Runs SMRT Link v13.1

Well		Run		Productivity				HiFi reads					Polymerase reads	Control reads		Library	File Transfer	
Plate well	Well name	Status	Movie time	Total bases	P0	P1	P2	Reads	Yield	Length (mean)	Read quality (medi...	Q30+ bases	Pol. read length (mean)	Reads	Read length (mean)	Missing adapter	Status	Action
1 D01	HiFi WGS Sample	Complete	24 hr	1,163 Gb	33%	67%	1%	6.4 M	104.1 Gb	16.4 kb	Q28	93%	69.0 kb	2,479	51.3 kb	7.0%	Complete	Retry File Transfer

Basic Preview (estimates)			Full Preview (estimates)			
Time (hr)	P1%	HiFi read length (mean)	Time (hr)	HiFi yield	HiFi read length (mean)	HiFi read quality (median)
4	64%	14.4 kb	23	97.1 Gb	16.1 kb	Q28

Column headers and reported metrics remain mostly **unchanged**[1]

Runs SMRT Link v25.1

Can optionally expand column headers to view other run QC metrics

Well		Run		HiFi reads					Productivity	Polymerase reads		Control reads		File transfer	
Plate well	Well name	Status	Movie time	Reads	Yield	Length (mean)	Read quality (median)	Q30+ bases	P1	Total bases	Pol. read length (mean)	Pol. read length (N50)	Reads	Read length (mean)	Status	Action
1 A01	HiFi WGS_Sample	Complete	24 hr	8.2 M	128.5 Gb	15.7 kb	Q34	93%	70%	1,348 Gb	76.2 kb	139.3 kb	501	65.5 kb	Complete	Retry File Transfer

Basic preview (estimates)			Full preview (estimates)			
Time	P1	HiFi read length (mean)	Time	HiFi yield	HiFi read length (mean)	HiFi read quality (median)
4 hr	65%	13.0 kb	23 hr	121.5 Gb	15.7 kb	Q33

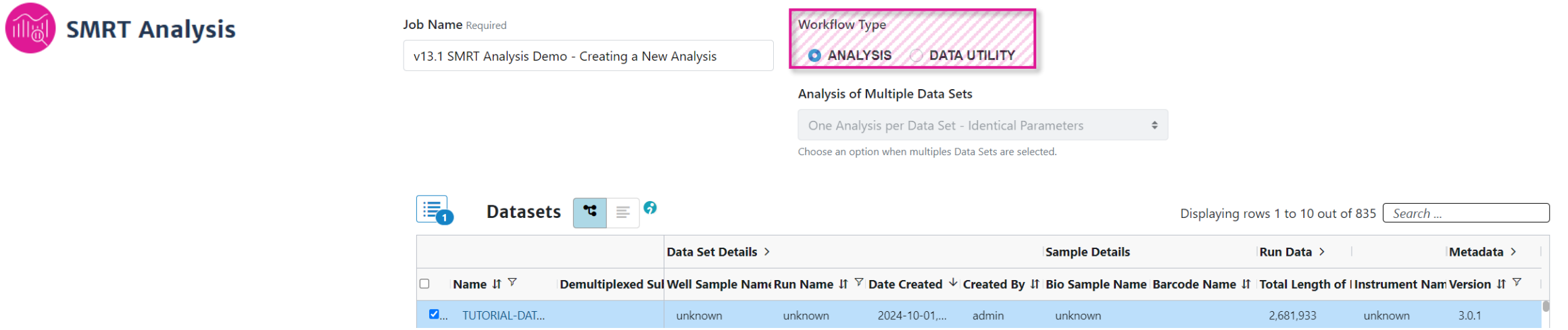
Productivity		Polymerase reads							
P1	Total bases	P0	P2	Pol. read length (mean)	Pol. read length (N50)	Longest subread (mean)	Longest subread (N50)	Base rate	Missing adapter
70%	1,348 Gb	29%	0%	76.2 kb	139.3 kb	16.8 kb	19.8 kb	2.2 bp/s	4.7%

[1] In SMRT Link v25.1, the default view for the **Productivity** tab shows **P1** and **Total bases**, and the **Missing adapter** metric has been moved under the **Polymerase reads** tab.

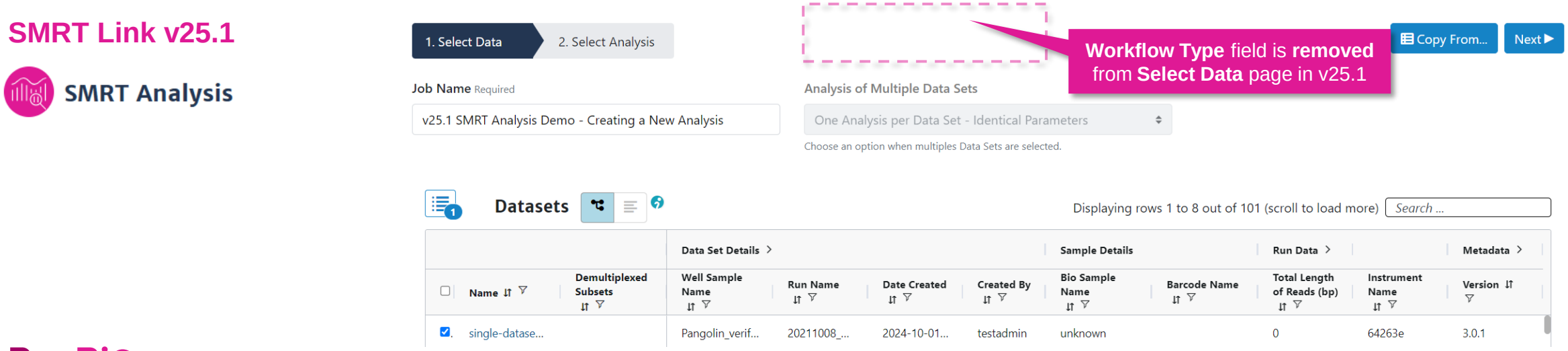
SMRT Link v25.1 SMRT Analysis user interface updates – Create New Job

Updated SMRT Analysis interface enables more streamlined job creation workflow

SMRT Link v13.1



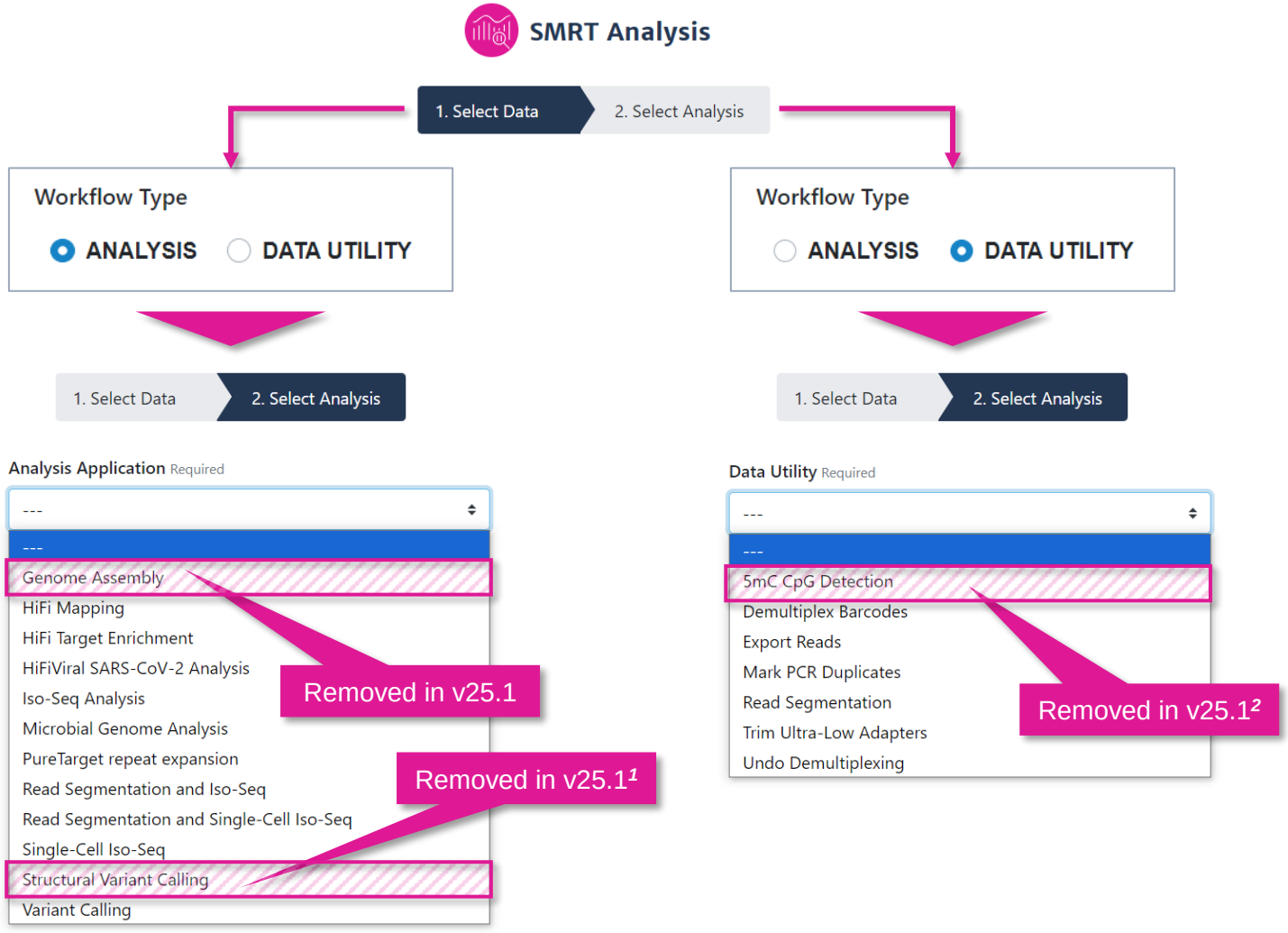
SMRT Link v25.1



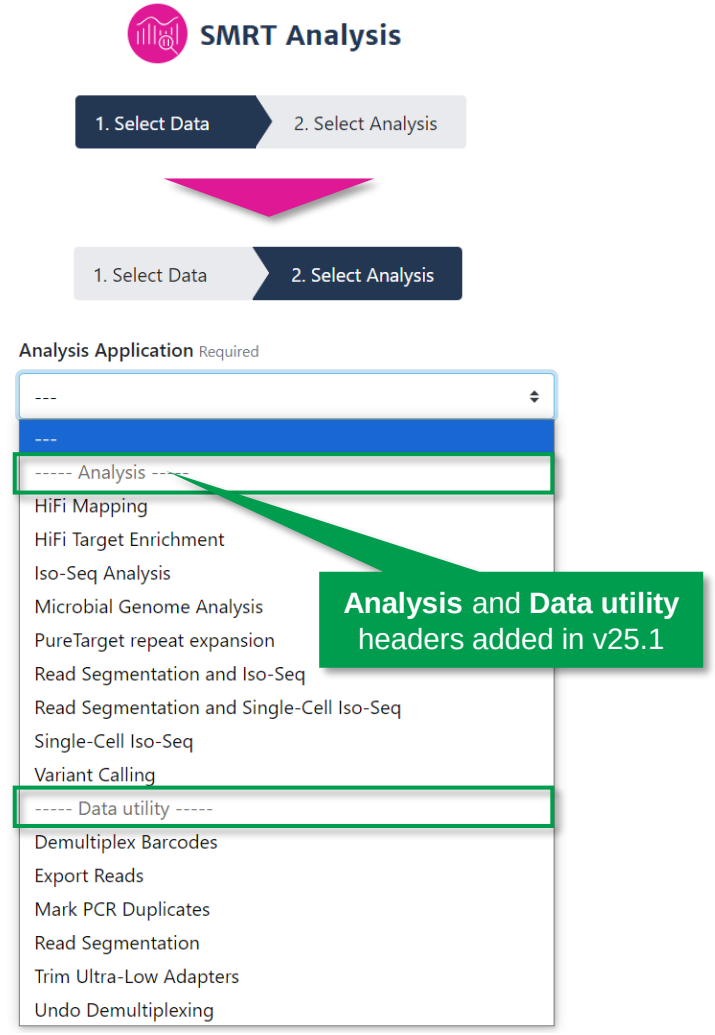
SMRT Link v25.1 SMRT Analysis user interface updates – Create New Job (cont.)

Updated SMRT Analysis interface enables more streamlined job creation workflow

SMRT Link v13.1



SMRT Link v25.1



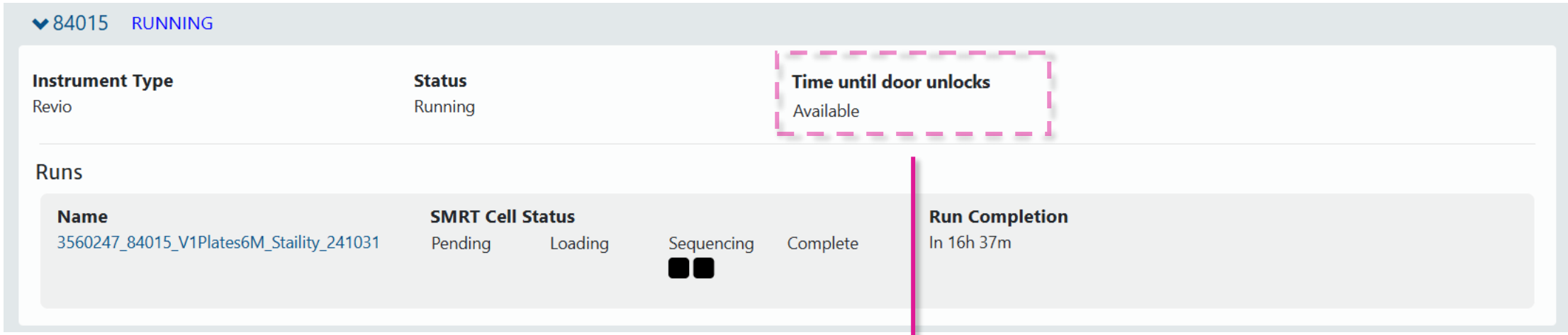
[1] SMRT Link v25.1 Variant Calling application includes structural variant (SV) calling analysis.

[2] 5mC calling feature is only available on-instrument in Revio system ICS v13.3 and is not available for off-instrument analysis in SMRT Link v25.1 user interface under Data Utilities. If needed, however, users can still use the command line tool version of jasmine available through the PacBio GitHub website at https://github.com/PacificBiosciences/jasmine

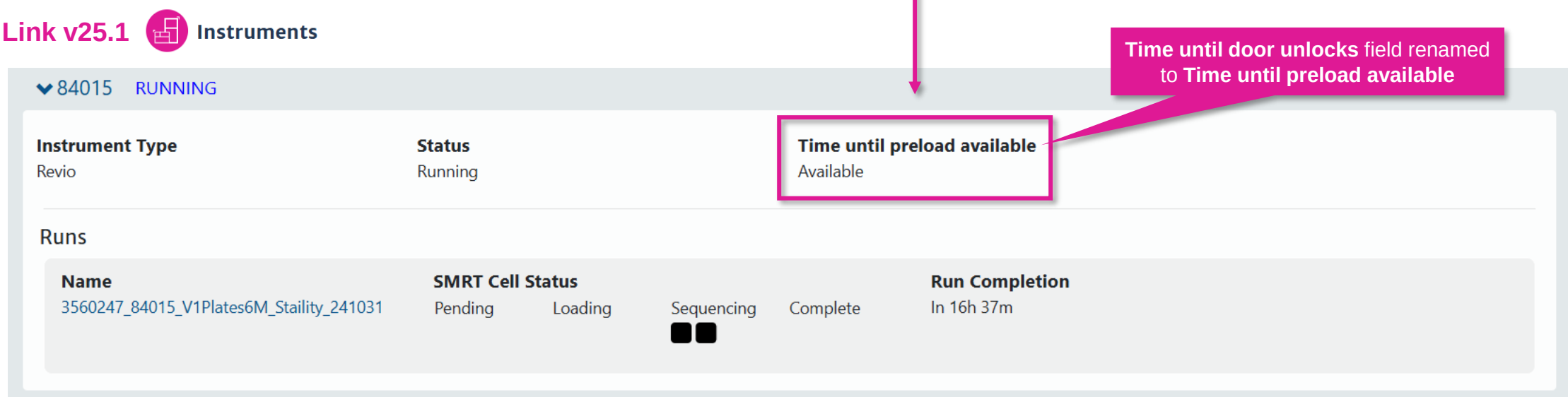
SMRT Link v25.1 Instruments module user interface updates

Minor update to Instruments home screen (Run Preview metrics and other features remain the same)

SMRT Link v13.1 Instruments



SMRT Link v25.1 Instruments



Time until door unlocks field renamed to **Time until preload available**

Revio system v13.3 applications & protocol updates

Revio system v13.3 supported applications & protocols

SPRQ chemistry is compatible with all SMRTbell prep kit 3.0, HiFi prep/plex kit 96, Kinnex, and PureTarget protocols

Sequencing method	Application	Protocol or Guide[1]
Whole genome sequencing	Large genome & small (microbial) genome WGS[2]	Preparing whole genome and metagenome libraries using SPK 3.0 [102-166-600] **[UPDATED]**
		Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system [103-424-100] **[UPDATED]**
		Preparing whole genome libraries using the HiFi prep kit 96 [103-420-700] **[UPDATED]**
		Automated HiFi prep 96 and HiFi ABC for the Hamilton NGS STAR MOA system [103-425-700] **[UPDATED]**
		Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800] **[UPDATED]**
		Automated HiFi plex prep 96 for the Hamilton NGS STAR MOA system [103-425-800] **[UPDATED]**
	Ultra-Low DNA input WGS	Preparing HiFi SMRTbell Libraries from Ultra-Low DNA Input [101-987-800] [Update TBD]
RNA sequencing	Kinnex full-length RNA	Preparing Kinnex libraries using Kinnex single-cell RNA kit [103-254-300] **[UPDATED]**
	Kinnex single-cell RNA	Preparing Kinnex libraries using Kinnex full-length RNA kit [103-238-700] **[UPDATED]**
Targeted sequencing	Amplicon sequencing	Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 [102-359-000] **[UPDATED]**
		Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers & SMRTbell prep kit 3.0 [101-921-300] [Update TBD]
		Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800] **[UPDATED]**
	HiFi target enrichment sequencing	Preparing multiplexed amplicon libraries using SPK 3.0 [102-359-000] **[UPDATED]**
	PureTarget sequencing	Generating PureTarget repeat expansion panel libraries [103-329-400] **[UPDATED]**
Viral sequencing	Adeno-associated virus (AAV)	Preparing multiplexed AAV SMRTbell libraries using SPK 3.0 [102-126-400] **[UPDATED]**
Metagenomics	Shotgun metagenomic assembly	Preparing whole genome and metagenome libraries using SPK 3.0 [102-166-600] **[UPDATED]**
	Shotgun metagenomic profiling	Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800] **[UPDATED]**
	Kinnex full-length 16S	Preparing Kinnex libraries from 16s rRNA amplicons [103-238-800] **[UPDATED]**

PacBio

1 **[UPDATED]** = Procedure & checklist is updated to support reduced DNA input requirements for SMRTbell library preparation and/or to include sample setup ABC guidance for Revio SPRQ chemistry.

2 Includes human/animal/plant/other WGS, microbial WGS, and shotgun metagenomic profiling or assembly.

Revio system v13.3 whole genome sequencing application updates

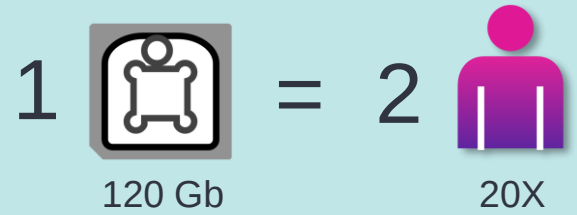
Revio SPRQ chemistry enables 2 human genomes to be sequenced to 20X coverage per SMRT Cell[1]

20× Human WGS

High accuracy SNV, SV calling performance at lower cost per genome; higher throughput + 5mC

- Population genomics (saliva samples)
- Disease cohort studies
- Screening, polygenic risk scores
- Complex disease profiling
- Clinical research

With Revio system SPRQ chemistry



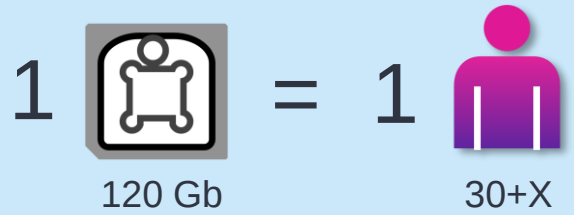
1,250 SMRT Cells/yr → 2,500 samples/yr[2]

30+× Human WGS

Improved power to detect and discover novel variants, now with lower input DNA requirements

- Rare disease studies / NICU (blood samples)
- Reference-grade *de novo* assembled genomes
- *De novo* mutation detection
- Population reference genomes
- Genome benchmarking
- Tumor sequencing

With Revio system SPRQ chemistry



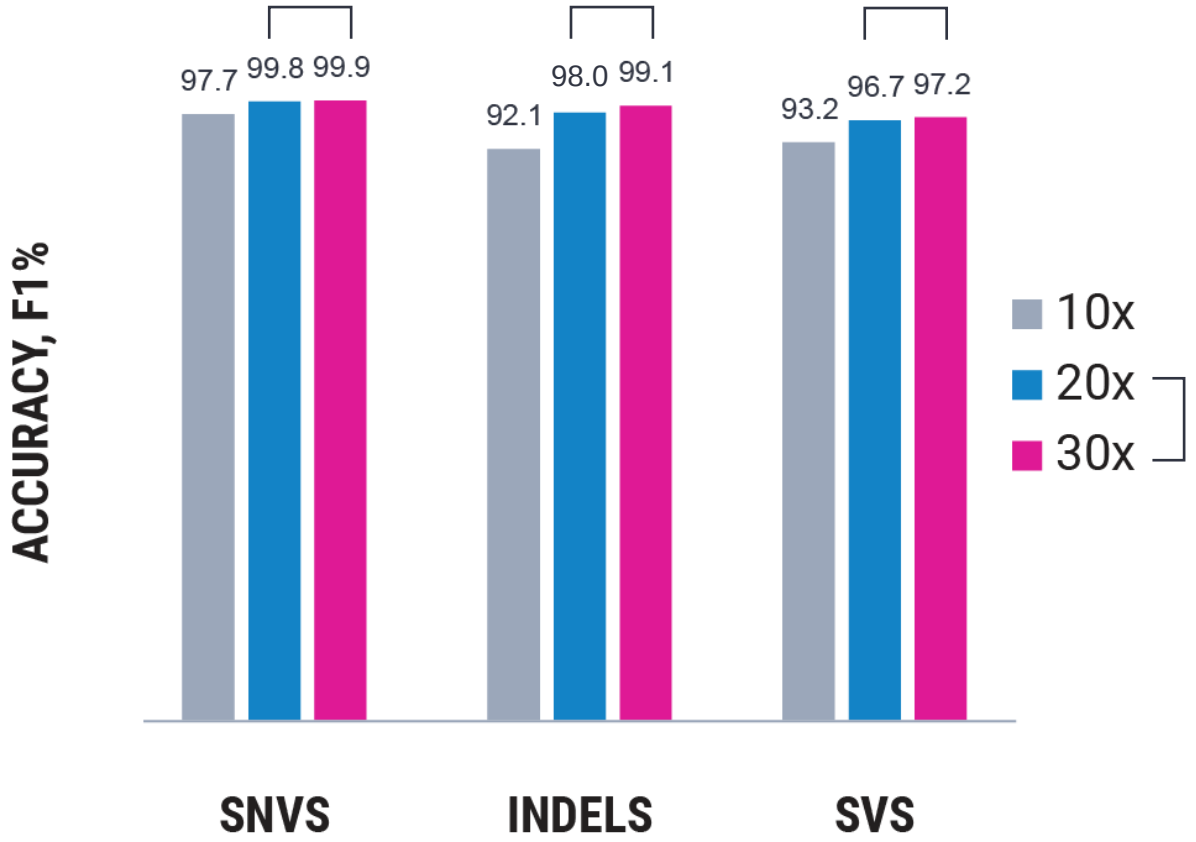
1,250 SMRT Cells/yr → 1,250 samples/yr[2]

PacBio

[1] Coverage may vary based on sample quality, library quality, and fragment lengths.
[2] Annual throughput is estimated and based on 1,250 Revio SMRT Cells for 24 hr runs.

Revio system v13.3 whole genome sequencing application updates (cont.)

20X HiFi WGS coverage approaches variant detection accuracy at 30X HiFi WGS coverage[1] with significant cost savings



Data shown is for a single Revio SMRT Cell for HG002/GM24385 sequenced with SPRQ chemistry.[2]

PacBio

[1] See ***Revio system brochure*** (102-326-543).
[2] F1 score = 2 * (Precision * Recall) / (Precision + Recall)

Revio system v13.3 Kinnex application updates

Revio SPRQ chemistry allows for higher data throughput and/or higher sample multiplexing[1] for Kinnex applications

		Kinnex single-cell RNA kit	Kinnex full-length RNA kit	Kinnex 16S RNA kit
				
Example application		Cell-type specific isoform discovery	Full-length isoform discovery	Full-length 16S rRNA for species identification
Throughput (per SMRT Cell)	Sequel II/IIe	30-40M	15-20M	20-25M
	Revio	80-100M	30-40M	50-60M
	Revio SPRQ	**100-120M**	**50-60M**	**60-80M**
Sample multiplexing	Sequel II/IIe	1-plex	3-plex (5M reads per sample)	384-plex
	Revio	For 10,000 unique reads per single cell: → 1-plex for 5,000 – 10,000 cells input → 2-plex for <5,000 cells input	4-plex (10M reads per sample)	384- to 1536-plex
	Revio SPRQ	For 12,000 unique reads per single cell: → 1-plex for 5,000 – 10,000 cells input → 2-plex for <5,000 cells input	**6-plex (10M reads per sample)** **12-plex (5M reads per sample)**	384- to 1536-plex

[1] With Revio SPRQ chemistry Kinnex library preparation procedures can typically provide sufficient library material to run 4 or more Revio SMRT Cells, enabling more flexibility with pooling options.

Revio system v13.3 PureTarget sequencing application updates

Revio SPRQ achieves excellent PureTarget coverage performance with reduced DNA sample input amounts

PureTarget application specifications	Revio	Revio SPRQ
DNA input	2 μg/sample (or 100 μg total per pool)	**1 μg/sample (or up to 50 μg total per pool)[1]**
DNA quality	GQN at 30 kb>5	GQN at 30 kb>5
Mean target coverage	>200-fold	>200-fold
Minimum target coverage	50-fold	50-fold
Sample multiplexing	48	48
Library size	4 – 5 kb	4 – 5 kb
Methylation	Detected	Detected

PureTarget™

[1] Using >50 μg of total input gDNA per pool may increase level of off-target reads. See ***Procedure & checklist – Generating PureTarget repeat expansion panel libraries*** (103-329-400) for the latest input gDNA mass recommendations for PureTarget library construction.

Revio system v13.3 HiFi library preparation protocol key updates

Sequencing method	Protocol or Guide[1]	Input sample QC[2]	Short read eliminator	DNA shearing	Library construction	ABC[3]
Whole genome sequencing	Preparing whole genome and metagenome libraries using SPK 3.0 [102-166-600]	Low mass input option	Low mass SRE option	Low mass shearing option	**No major changes**	New ABC section
	Short Read Eliminator (SRE), DNA shearing, and cleanup for the Hamilton Microlab Prep system [103-424-100]	—	Low mass SRE option	Low mass shearing option	—	—
	Preparing whole genome libraries using the HiFi prep kit 96 [103-420-700]	Low mass input option	Low mass SRE option	Low mass shearing option	**No major changes**	Updated ABC section
	Automated HiFi prep 96 and HiFi ABC for the Hamilton NGS STAR MOA system [103-425-700]	Low mass input option	Low mass SRE option	Low mass shearing option	**No major changes**	Updated ABC section
	Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800]	**No major changes**	—	**No major changes**	Updated post-nuclease cleanup pooling options	New ABC section
	Automated HiFi plex prep 96 for the Hamilton NGS STAR MOA system [103-425-800]	**No major changes**	—	**No major changes**	Updated post-nuclease cleanup pooling options	New ABC section
RNA sequencing	Preparing Kinnex libraries using Kinnex single-cell RNA kit [103-254-300]	**No major changes**	—	—	**No major changes**	New ABC section
	Preparing Kinnex libraries using Kinnex full-length RNA kit [103-238-700]	**No major changes**	—	—	Updated post-nuclease cleanup elution volume	New ABC section
Targeted sequencing	Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 [102-359-000]	**No major changes**	—	—	Added table for [DNA] normalization before ABC	New ABC section
	Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800]	**No major changes**	—	**No major changes**	Updated post-nuclease cleanup pooling options	New ABC section
	Generating PureTarget repeat expansion panel libraries [103-329-400]	Added table to clarify input req.	—	—	**No major changes**	Updated ABC section
Viral sequencing	Preparing multiplexed AAV SMRTbell libraries using SPK 3.0 [102-126-400]	**No major changes**	—	—	**No major changes**	New ABC section
Metagenomics	Preparing whole genome and metagenome libraries using SPK 3.0 [102-166-600]	Low mass input option	Low mass SRE option	Low mass shearing option	**No major changes**	New ABC section
	Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800]	**No major changes**	—	**No major changes**	Updated post-nuclease cleanup pooling options	New ABC section
	Preparing Kinnex libraries from 16s rRNA amplicons [103-238-800]	**No major changes**	—	—	Updated post-nuclease cleanup elution volume	New ABC section

PacBio

1 Refer to PacBio Documentation website for the most up-to-date versions of Procedure & checklist and Guide & overview SMRTbell library preparation documentation.
2 Recommended QC methods and tools to evaluate input gDNA sample quality remain the same for all protocols – but some protocols have updated minimum input gDNA mass requirements.
3 **ABC** = Annealing / binding / cleanup step to prepare purified, primer-annealed and polymerase-bound SMRTbell libraries for sequencing on PacBio long-read systems.

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]

QC
- Input DNA sample QC

SRE
- 1 Short Read Eliminator on gDNA

DNA Shearing
- 2 Pipette DNA shearing
- 3 Post-shearing cleanup

WGS library construction
- 4 DNA repair & A-tailing
- 5 Adapter ligation & cleanup
- 6 Nuclease treatment
- 7 AMPure PB cleanup and size selection

ABC
- 8 Annealing, binding & cleanup

gDNA input mass into library prep	Sequel II® and Sequel IIe	Revio™ non-SPRQ™/Vega	Revio SPRQ™
Total DNA per SMRT® Cell*	1 µg per SMRT® Cell 8M	2 µg per Revio™ SMRT® Cell	500 ng per Revio™ SMRT® Cell

- New **low mass input** option available with updated parameters for SRE & DNA shearing when using Revio SPRQ with **0.5 – 1.25 µg of input gDNA**[1]

✔	Step	Instructions
	1.1	Dilute gDNA to the appropriate concentration in Buffer LTE. The gDNA input mass into SRE will depend on the expected recovery. If expected SRE recovery is ≤1 µg, follow "Low mass" guidelines. If expected SRE recovery is >1µg – 3 µg, follow "High mass" guidelines (see Table 1 for expected recoveries based on DNA quality). This ensures that the DNA concentration will be within the correct range for the subsequent pipette shearing step.

- **Low mass SRE** method has been optimized to provide comparable recoveries to high mass SRE with the same size selection performance
- High mass and low mass samples **cannot** be included in the **same** SRE automation run

✔	Step	Instructions
	2.1	Adjust DNA concentration to ≤5 ng/µL in 200 µl or ≤10 ng/µL in 300 µl, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use the Low TE buffer provided with the kit (or buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).

- **Low mass shearing** method has been optimized to maintain the size distribution between 15 – 20 kb
- High mass and low mass samples **cannot** be included in the **same** shearing automation run

No major changes to SPK 3.0 WGS library construction steps

✔	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**
- Recommend to **pool adapter-indexed WGS libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

[1] If 0.5 – 1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the low mass workflow will provide enough library for one Revio SPRQ SMRT Cell run; however, high mass parameters can also be used for Revio SPRQ if excess library is desired. For Revio non-SPRQ chemistry and Vega, 2 µg gDNA input is still recommended for loading of 1 SMRT Cell.

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates (cont.)

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]

QC
- Input DNA sample QC

SRE
- 1 Short Read Eliminator on gDNA

DNA Shearing
- 2 Pipette DNA shearing
- 3 Post-shearing cleanup

WGS library construction
- 4 DNA repair & A-tailing
- 5 Adapter ligation & cleanup
- 6 Nuclease treatment
- 7 AMPure PB cleanup and size selection

ABC
- 8 Annealing, binding & cleanup

gDNA input mass into library prep	Sequel II® and Sequel IIe	Revio™ non-SPRQ™/Vega	Revio SPRQ™
Total DNA per SMRT® Cell*	1 µg per SMRT® Cell 8M	2 µg per Revio™ SMRT® Cell	500 ng per Revio™ SMRT® Cell

DNA quality recommendation	Femto Pulse genome quality score	
DNA size distribution (Femto Pulse system)	70% ≥10 kb (GQN_{10kb} ≥7.0) & 50% ≥30 kb (GQN_{30kb} ≥5.0)	Lower quality DNA may be used with the expectation of lower sequencing yields.

gDNA quality	≤1.25 µg (Revio SPRQ)	≥2 µg	Expected SRE recovery (dependent on DNA quality)
70% > 10 kb	0.5 – 1.25 µg	2 – 4 µg	60 – 95%
<70% > 10kb	1 – 1.75 µg	4 – 5 µg	40 – 60%
<10kb (no SRE)	0.25 – 1 µg	1 – 3 µg	Not recommended

DNA fragment size recommendations	
DNA shearing	Automated pipette-tip shearing
Target fragment lengths	15–20 kb
Size selection	Short read eliminator on gDNA, and 3.1X (35% v/v) AMPure® PB on HiFi library

Protocol Step	DNA or SMRTbell step recovery	DNA or SMRTbell overall recovery
Starting Input	100%	100%
Post-SRE	65-95%	65-95%
Post-shear SMRTbell bead cleanup	80-95%	52-90%
Post-ligation SMRTbell bead cleanup	80-95%	42-86%
Post-nuclease (pre-cleanup)	40-50%	17-43%
Post-3.1x AMPure PB bead cleanup	75-80%	13-34%
Post-ABC cleanup	75-95%	10-32%

Mean insert size	Revio SPRQ (250 pM)	Revio non-SPRQ/Vega (250 pM)
10,000 bp	41 ng	163 ng
15,000 bp	61 ng	244 ng
18,000 bp	73 ng	293 ng
21,000 bp	85 ng	341 ng

Input DNA sample QC notes

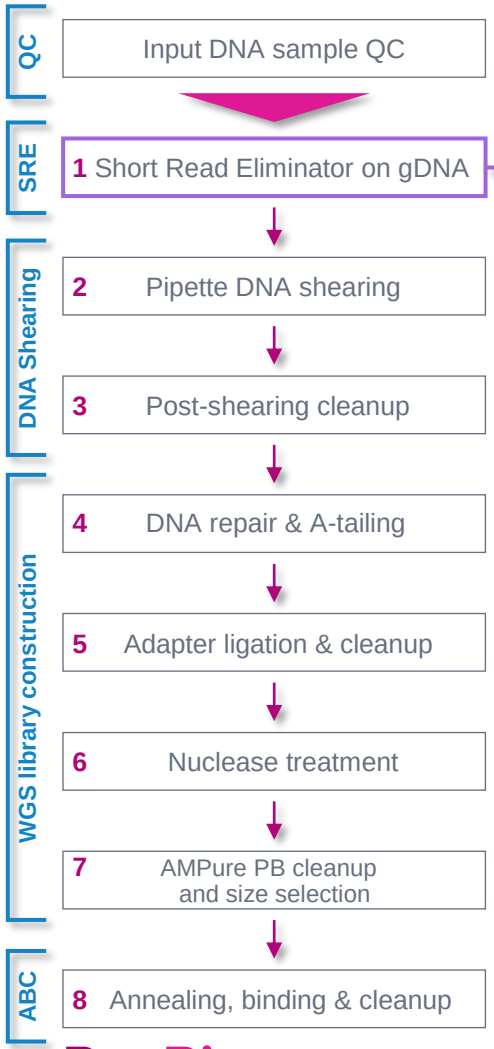
- Starting with **500 ng** and 2 µg of genomic DNA will typically provide enough library to load at least **1 Revio SPRQ SMRT Cell** and 1 Revio non-SPRQ/Vega SMRT Cell, respectively[1]
- **Input DNA QC recommendations are the same** for Revio SPRQ & non-SPRQ libraries
  - ≥70% of starting input gDNA should be ≥10 kb (GQN_{10kb} ≥ 7.0. If GQN_{10kb} <7.0, higher gDNA inputs may be required
- **If starting with lower quality gDNA, using a higher input amount >500 ng is recommended**
- **Target DNA fragment size (15 – 20 kb) is the same** for Revio SPRQ & non-SPRQ libraries
  - Shearing may be bypassed if sample is already in the appropriate size-range
  - If majority of DNA is <10 kb, SRE is not recommended
- Overall recovery is dependent on gDNA quality and size
  - **Overall recovery from input gDNA to post-ABC cleanup ranges between ~10 – 32%**
- Min. polymerase-bound library mass necessary for loading 1 Revio SPRQ SMRT Cell (250 pM) ranges **~41 ng to 85 ng** depending on insert size

[1] If 0.5 – 1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the low mass workflow will provide enough library for one Revio SPRQ SMRT Cell run; however, high mass parameters can also be used for Revio SPRQ if excess library is desired. For Revio non-SPRQ chemistry and Vega system libraries, 2 µg gDNA input is still recommended for loading of 1 SMRT Cell.

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates (cont.)

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]



✔	Step	Instructions
	1.1	Dilute gDNA to the appropriate concentration in Buffer LTE. The gDNA input mass into SRE will depend on the expected recovery. If expected SRE recovery is ≤1 µg, follow "Low mass" guidelines. If expected SRE recovery is >1µg – 3 µg, follow "High mass" guidelines (see Table 1 for expected recoveries based on DNA quality). This ensures that the DNA concentration will be within the correct range for the subsequent pipette shearing step.

	Low mass	High mass
Sample volume	25 µL	50 µL
DNA concentration	20–50 ng/µL	40–80 ng/µL
Recommended max gDNA mass	1.25 µg*	4 µg**
Elution volume (Buffer LTE)	200 µl	300 µl
Shearing mass limit	≤1 µg	≤3 µg

*Max gDNA input mass is 1.75 µg for low quality gDNA with a GQN10kb <7.0. See Table 1.

** Max gDNA input mass is 5 µg for low quality gDNA with a GQN10kb <7.0. See Table 1.

1.2 Add **Buffer SRE** to each sample.

	Low mass	High mass
Buffer SRE volume	25 µL	50 µL

If working in a plate format, heat seal with foil. Vortex/shake to mix for 5 seconds at max speed.

1.6 Carefully remove supernatant without disturbing the pellet.

- Leaving up to 5 µl and 10 µl supernatant with the low and high mass SRE workflow, respectively, is acceptable to ensure the pellet is not aspirated.

1.7 Add **Buffer LTE** to the tube and incubate at room temperature for 10 minutes.

	Low mass	High mass
Buffer LTE	200 µL	300 µL

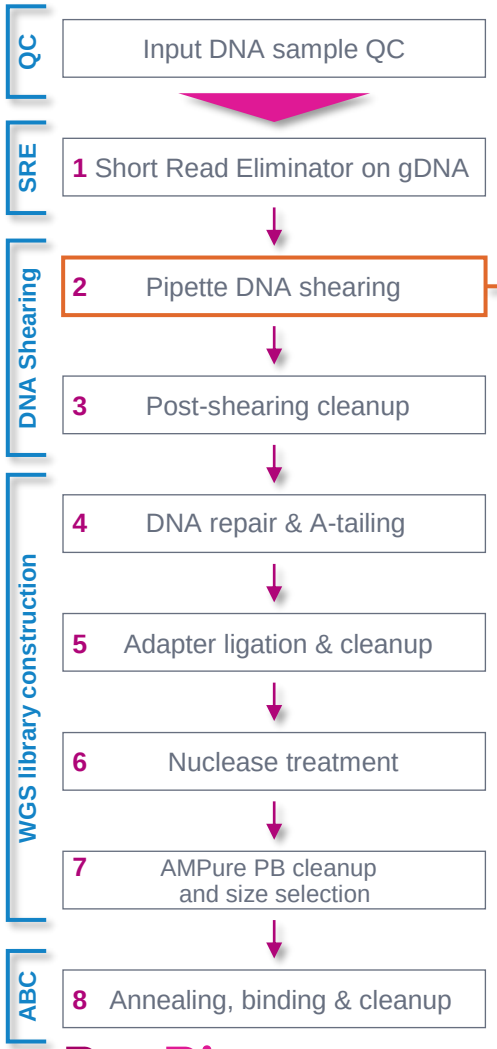
Short read eliminator (SRE) notes

- New **low mass** option available with updated parameters for SRE when using Revio SPRQ with **0.5 – 1.25 µg of input gDNA**
- **Low mass SRE is optimized** to provide comparable recoveries to high mass SRE with the same size selection performance
- Recommend to **stay consistent for both SRE and shearing steps** (i.e., use low or high mass settings for both SRE and shearing)
- **Note: Both high mass and low mass samples cannot be included in the same SRE automation run**
- **Important: Use SRE on genomic DNA only.** Attempting to use SRE on sheared DNA or HiFi libraries will result in poor recoveries
- If automating this step, refer to ***Microlab Prep system Guide & overview*** (103-424-100) for details on consumables
- Recommend to proceed to DNA shearing within 2 weeks of performing SRE
- If not performing SRE, proceed directly to the DNA shearing step

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates (cont.)

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]



✔	Step	Instructions
	2.1	Adjust DNA concentration to **≤5 ng/µL in 200 µl or ≤10 ng/µL in 300 µl**, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use the Low TE buffer provided with the kit (or buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).
	2.2	Parameters for shearing on the Microlab Prep, or Hamilton assay-ready workstations are listed below. These parameters should already be part of the installed method on the instrument.
	2.3	Place the plate on the appropriate work deck position and start the shearing procedure.
	2.4	Optional: measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit once the shearing procedure is complete. **Recommended**: Further dilute each aliquot to **250 pg/µL** with the Femto Pulse dilution buffer. Measure the DNA with a Femto Pulse system to ensure efficient shearing.
	2.5	Proceed to the next step of the protocol.

Parameter	Low mass	High mass
DNA concentration	≤5 ng/µL	≤10 ng/µL
Volume of Buffer LTE	200 µL	300 µL
Number of mixes	300 cycles	300 cycles
Pipette mixing speed	400 µL/sec	500 µL/sec
Liquid following	83% volume	83% volume
Pipette tip	300 µL CO-RE II tips (filtered, black, non-sterile)	300 µL CO-RE II tips (filtered, black, non-sterile)

DNA shearing notes

- New **low mass** option available with updated parameters for DNA shearing when using Revio SPRQ with **0.5 – 1.25 µg of input gDNA**
- **Low mass shearing settings are optimized** to maintain the size distribution between **15 – 20 kb**, which is this recommended target fragment size range for this WGS protocol
- Section 2 of protocol describes the procedure for DNA shearing with the **Hamilton Microlab Prep or Hamilton assay ready workstations** (NGS STAR MOA, STARlet, and STAR V).[1]
- See protocol Appendix for instructions on shearing with the **Megaruptor 3 system**
- Deviating from the stated concentration and automation settings is not recommended and will result in under-sheared DNA
- Sheared DNA fragment size distribution should **ideally be narrow and generally between 10 to 30 kb**
- If input gDNA is already within these ranges or lower, DNA shearing step can be bypassed

[1] It may be possible to shear DNA using other NGS liquid handler systems. Please check with your local PacBio support team for updated information on all qualified DNA shearing methods.

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates (cont.)

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]

QC
- Input DNA sample QC

SRE
- 1 Short Read Eliminator on gDNA

DNA Shearing
- 2 Pipette DNA shearing
- 3 Post-shearing cleanup

WGS library construction
- 4 DNA repair & A-tailing
- 5 Adapter ligation & cleanup
- 6 Nuclease treatment
- 7 AMPure PB cleanup and size selection

ABC
- 8 Annealing, binding & cleanup

✔	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔ Tube	Component	Volume
Light blue	Annealing buffer	12.5 µL
Light green	Standard sequencing primer	12.5 µL
	Total volume	**25 µL**

Annealing step

	8.2	Pipette-mix the **Annealing mix** and quick spin to collect liquid.
	8.3	Add **25 µL** of **the Annealing mix** to each library. Total volume should equal **50 µL.**
	8.7	To prepare the polymerase, add the following components to a new microcentrifuge tube on ice. Adjust component volumes for the number of samples being prepared, plus 10% overage.

Polymerase Dilution

✔ Tube	Component	Volume
Yellow	Polymerase buffer	47 µL
Purple	Sequencing polymerase	3 µL
	Total volume	**50 µL**

Binding step

	8.8	Pipette mix the **polymerase dilution** and quick-spin to collect liquid.
	8.9	Add **50 µL of polymerase dilution** to primer annealed sample. Total volume should equal **100 µL**.

Sample setup ABC notes

- To prepare **Revio SPRQ libraries** for sequencing, after completing AMPure PB bead cleanup and size selection (Step 7), **proceed to Step 8 in protocol for sample setup ABC instructions[1] (do not use SMRT Link ABC calculator tool)**
- Section 8 describes annealing, binding & cleanup procedure using **static reagent and buffer volumes** for a starting library sample that contains **<60 ng/µL of DNA in 25 µL of EB**
  - **No requirement to adjust reagent and buffer volumes according library DNA concentration or library insert size**
  - If a custom sample volume is used, see protocol Appendix to prepare required amounts of Annealing mix and polymerase dilution
- Recommend to **pool adapter-indexed WGS libraries post-ABC**
  - If samples are pooled prior to ABC see protocol Appendix to prepare required amounts of Annealing mix and polymerase dilution
- Proceed to perform post-binding cleanup with **1X SMRTbell cleanup beads**

[1] To prepare Revio non-SPRQ libraries for sequencing, after completing AMPure PB bead cleanup and size selection (Step 7), use SMRT Link Sample Setup module for performing sample setup ABC.

Revio system v13.3 SMRTbell prep kit 3.0 WGS protocol updates (cont.)

SPK 3.0 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 [102-166-600 Rev 05]

QC
- Input DNA sample QC

SRE
- 1 Short Read Eliminator on gDNA

DNA Shearing
- 2 Pipette DNA shearing
- 3 Post-shearing cleanup

WGS library construction
- 4 DNA repair & A-tailing
- 5 Adapter ligation & cleanup
- 6 Nuclease treatment
- 7 AMPure PB cleanup and size selection

ABC
- 8 Annealing, binding & cleanup

✔	Step	Instructions
		Post-binding cleanup with 1X SMRTbell cleanup beads
	8.13	Add **100 µL** of resuspended, room-temperature SMRTbell cleanup beads to each sample
	8.14	Pipette-mix the beads until evenly distributed and quick-spin if necessary to collect all liquid from the sides of the tube.
	8.15	Incubate at **room temperature** for **10 minutes** to allow DNA to bind beads
	8.16	Place sample on an appropriate magnet and allow beads to separate fully from the solution
	8.17	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant. **DO NOT USE EtOH.** Proceed immediately to the elution. It is important not to let the beads dry out.
	8.18	Remove sample from the magnet and **immediately** add **Loading buffer** to each tube and resuspend the beads by pipette mixing.
	8.19	Quick-spin the samples to collect any liquid from the sides of the tube.
	8.20	Incubate at **room temperature** for 15 **minutes** to elute DNA
	8.21	Place sample on magnet and allow beads to separate fully from the solution.
	8.22	Slowly remove the cleared eluate without disturbing the beads. Transfer eluate to a **new tube**. Discard the old tube with beads
	8.23	Use **1 µL** of sample to measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. **Important:** The **Qubit Flex** instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96. Concentration readings will not be accurate.
	8.24	Proceed to the **Loading Calculator** in SMRT Link v13.3 or higher to calculate the final dilution for adding the sample to Sequencing reagent plate. The recommended loading concentration is 200 – 300 pM. Polymerase-bound libraries can be stored at 4°C for 1 month, or at -20°C for >6 months prior to sequencing. Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles without affecting sequencing performance.

	Revio SPRQ	Vega
Loading buffer	25 µL	50 µL

Cleanup step

PROTOCOL COMPLETE

Sample setup ABC notes (cont.)

- Perform post-binding cleanup with **1X SMRTbell cleanup beads**[1]
- Elute bound sample from SMRTbell cleanup beads using **25 µL of Loading buffer** if using Revio SPRQ polymerase
- Transfer eluate to a new tube and **use 1 µL of sample to measure DNA concentration** with a Qubit fluorometer using the 1x dsDNA HS kit.
  - Important: The **Qubit Flex instrument is not compatible with measuring polymerase-bound library in Loading Buffer 96**. Concentration readings will not be accurate
- Polymerase-bound libraries can be stored at 4°C for 1 month, or at -20°C for >6 months prior to sequencing
- Polymerase-bound libraries can withstand up to 4 freeze-thaw cycles without affecting sequencing performance

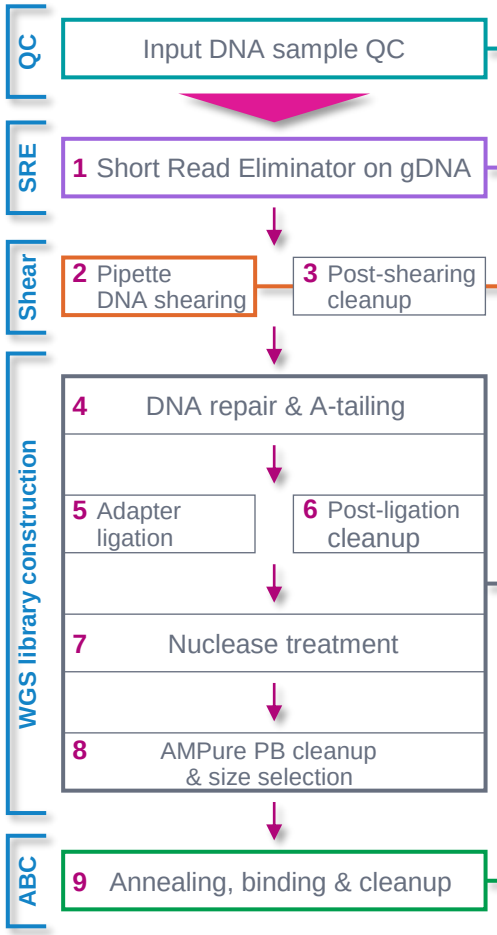
- After ABC, proceed to the **Loading Calculator in SMRT Link v25.1 to prepare DNA internal control and final loading dilution**
  - Recommended on-plate loading concentration for 15-20 kb WGS libraries is **200 – 300 pM**

[1] A 1.0X SMRTbell cleanup bead ratio is recommended for library insert sizes 15 – 20 kb. Perform the post-binding cleanup step using the specific SMRTbell cleanup bead ratio stated in the relevant application-specific Procedure & checklist being followed.

Revio system v13.3 HiFi prep kit 96 WGS protocol updates

HiFi prep kit 96 whole genome sequencing protocol supports reduced DNA input requirements for SMRTbell library preparation and include updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing whole genome libraries using the HiFi prep kit 96 [103-420-700 Rev 03]



DNA input mass and fragment size recommendations

gDNA per SMRT® Cell	Revio non-SPRQ/Vega: 2 µg Revio SPRQ: 500 ng

- New **low mass input** option available with updated parameters for SRE & DNA shearing when using Revio SPRQ with **0.5 – 1.25 µg of input gDNA**[1]

✔	Step	Instructions for SRE on gDNA
		Dilute gDNA to the appropriate concentration in Buffer LTE. The gDNA input mass into SRE will depend on the expected recovery. If expected SRE recovery is ≤1 µg, follow "Low mass" guidelines. If expected SRE recovery is >1µg – 3 µg, follow "High mass" guidelines (see Table 1 for expected recoveries based on DNA quality). This ensures that the DNA concentration will be within the correct range for the subsequent pipette shearing step.

- **Low mass SRE** method has been optimized to provide comparable recoveries to high mass SRE with the same size selection performance
- High mass and low mass samples **cannot** be included in the **same** SRE automation run

✔	Step	Instructions for automated DNA shearing on Hamilton systems
	2.1	Adjust DNA concentration to **≤5 ng/µL in 200 µl or ≤10 ng/µL in 300 µl**, if necessary (e.g., if more than 1 µg or 3 µg of gDNA was recovered from SRE). Use the **Low TE buffer** provided with the kit (or buffer LTE from the SRE kit) to dilute samples in a 0.8 mL, 96 DeepWell plate (Thermo Fisher Scientific AB0859).

- **Low mass shearing** method has been optimized to maintain the size distribution between 15 – 20 kb
- High mass and low mass samples **cannot** be included in the **same** shearing automation run

No major changes to HiFi prep kit 96 WGS library construction steps

✔	Step	Instructions
	9.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer 96	12.5 µL
	Light green	Standard sequencing primer 96	12.5 µL
		Total volume	**25 µL**

- Updated section (Step 9) describes **ABC procedure for Revio SPRQ and non-SPRQ samples using static reagent & buffer volumes**[2]
- Recommend to **pool adapter-indexed WGS libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

[1] If 0.5 – 1.25 µg gDNA is available and Revio SPRQ chemistry is being used, the low mass workflow will provide enough library for one Revio SPRQ SMRT Cell run; however, high mass parameters can also be used for Revio SPRQ if excess library is desired. For Revio non-SPRQ chemistry and Vega, 2 µg gDNA input is still recommended for loading of 1 SMRT Cell.

[2] After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (200 – 300 pM OPLC).

Revio system v13.3 HiFi plex prep kit 96 WGS protocol updates

HiFi plex prep kit 96 multiplexed whole genome sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800 Rev 03]

QC
- DNA sample QC

DNA shearing
- 1 Pipette DNA shearing
- 2 Post-shearing cleanup

Multiplexed WGS library construction
- 3 DNA repair & A-tailing
- 4 Indexed adapter ligation & pooling
- 5 Pool cleanup
- 6 Nuclease treatment (24-plex sample pool)
- 7 Final pooling & SMRTbell bead cleanup

ABC
- 8 Annealing, binding & cleanup

No major changes to input genomic DNA sample QC steps

gDNA	Amplicons
50–300 ng per sample	20–200 ng per sample

- Required input gDNA mass (**50 – 300 ng per sample**) remains the **same** for Revio SPRQ and non-SPRQ samples

7. Pooling and SMRTbell cleanup bead purification

Pool the completed nuclease reactions based on the number of SMRTcells to be run. For example, if 96 samples will be sequenced on a single SMRTcell, pool four 24-sample nuclease reactions prior to SMRTbell cleanup.

Nuclease reactions pooled	Total volume	SMRTbell cleanup bead volume for 1.0x	SMRTbell cleanup bead volume for 1.3x	Elution volume
1	50 µl	50 µl	65 µl	25 µl
2	100 µl	100 µl	130 µl	25 µl
3	150 µl	150 µl	195 µl	25 µl
4	200 µl	200 µl	260 µl	25 µl

Minor updates to HiFi plex prep kit 96 WGS library construction steps

- Added table to **clarify pooling options and SMRTbell cleanup bead volumes to use for post-nuclease cleanup step (Step 7)** [each nuclease reaction contains up to 24 adapter-indexed samples]1

✔	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer 96	12.5 µL
	Light green	Standard sequencing primer 96	12.5 µL
		Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[2, 3]
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

1 In the previous version of this procedure (103-418-800 Rev 02), each nuclease reaction (containing up to 24 pooled samples) is purified **individually** instead of being pooled together.
2 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 8 in protocol) for performing sample setup ABC.
3 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (200 – 300 pM OPLC).

Revio system v13.3 Kinnex single-cell RNA sequencing protocol updates

Kinnex single-cell RNA sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing Kinnex libraries using Kinnex single-cell RNA kit [103-254-300 Rev 06]

QC

1 Input cDNA sample QC

Kinnex library construction

2 TSO PCR

3 TSO artifact removal

4 Kinnex PCR & cleanup

5 Kinnex array formation & cleanup

6 DNA damage repair

7 Nuclease Tx & final cleanup

ABC

8 Annealing, binding & cleanup

No major changes to input cDNA sample QC steps

cDNA input	
Quantity	>15 ng per library cDNA concentration should be >1ng/µL with up to 15 µL in volume. See step 2.1 for 10x cDNA input requirement.

- Required input cDNA mass (**>15 ng per library**) remains the **same** for Revio SPRQ and non-SPRQ samples

No major changes to Kinnex single-cell RNA library construction steps

7. Nuclease treatment and cleanup

✔	Step	Instructions
	7.16	Remove the tube strip from the magnetic rack. Immediately add 26 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	7.22	Normalize 25 µl of library to 20 ng/µl prior to ABC. A concentration of less than 20 ng/µL is acceptable if sample quantity is limited. Using a concentration above 20 ng/µL will result in lower loading during sequencing.

- During post-nuclease cleanup (**Step 7**), **elute Kinnex single-cell RNA library in 26 µL EB and normalize 25 µL of library to 20 ng/µL prior to ABC**

✔	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Kinnex sequencing primer	12.5 µL
		Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[1, 2]
- Recommend to **pool adapter-indexed Kinnex libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

PacBio

1 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 8 in protocol) for performing sample setup ABC.
2 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (130 – 160 pM OPLC).

Revio system v13.3 Kinnex full-length RNA sequencing protocol updates

Kinnex full-length RNA sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing Kinnex libraries using Kinnex full-length RNA kit [103-254-300 Rev 06]

QC

1 Input RNA sample QC

Kinnex library construction

2 cDNA synthesis & cleanup

3 cDNA amplification & cleanup

4 Kinnex PCR & cleanup

5 Kinnex array formation & cleanup

6 Nuclease Tx & final cleanup

ABC

7 Annealing, binding & cleanup

No major changes to input RNA sample QC steps

RNA input	
Quality/size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300 ng per library (minimum concentration 43 ng/µL per library)

- Required input RNA mass (**>300 ng per library**) and RNA quality (**RIN ≥ 7.0**) remains the **same** for Revio SPRQ and non-SPRQ samples

6.2 Final cleanup with SMRTbell cleanup beads

✔	Step	Instructions
	6.2.9	Remove the tube strip from the magnetic rack. Immediately add 26 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.2.15	If required, normalize 25 µl of library to <60 ng/µl. Using a concentration above 60 ng/µl into ABC will result in poor sequencing performance.

Minor updates to Kinnex full-length RNA library construction steps

- During post-nuclease cleanup (**Step 6**), **elute Kinnex full-length RNA library in 26 µL EB (instead of 20 µL) and normalize 25 µL of library to <60 ng/µL prior to ABC**[1]

✔	Step	Instructions
	7.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Kinnex sequencing primer	12.5 µL
		Total volume	**25 µL**

- New section (Step 7) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[2,3]
- Recommend to **pool adapter-indexed Kinnex libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

PacBio

1 In the previous version of this procedure (103-254-300 Rev 05), Kinnex full-length RNA library is eluted in in **20 µL** EB and the DNA concentration must be less than 60 ng/µL to go into ABC.
2 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 7 in protocol) for performing sample setup ABC.
3 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (130 – 160 pM OPLC).

Revio system v13.3 Kinnex 16S sequencing protocol updates

Kinnex 16S sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing Kinnex libraries from 16S rRNA amplicons [103-238-800 Rev 04]

QC

1 16S FL sequence capture

Kinnex library construction

2 Pooling & SMRTbell cleanup

3 Kinnex PCR & cleanup

4 Kinnex array formation & DNA repair

5 SMRTbell cleanup

6 Nuclease treatment

7 Final SMRTbell bead cleanup

ABC

8 Annealing, binding & cleanup

No major changes to input genomic DNA sample QC steps

DNA input

To ensure pipetting accuracy, plan to deliver 1–2 ng of gDNA to each individual PCR reaction in a constant 5 µL volume. Normalize sample gDNA concentration to 0.2–0.4 ng/µL in 10 mM Tris-HCl pH 8.0–8.5 (elution buffer) prior to setting up PCR reactions. The recommended total input gDNA per reaction is 1–2 ng.

- Required input gDNA amount (**1 – 2 ng per sample**) remains the **same** for Revio SPRQ and non-SPRQ samples

7. Final cleanup with SMRTbell cleanup beads

Step	Instructions
7.9	Remove the tube strip from the magnetic rack. Immediately add 26 µL of Elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
7.15	If required, normalize 25 µl of library to <60 ng/µl. Using a concentration >60 ng/µL will result in poor sequencing performance.

Minor updates to Kinnex 16S library construction steps

- During post-nuclease cleanup (**Step 7**), **elute Kinnex 16S RNA library in 26 µL EB (instead of 20 µL) and normalize 25 µL of library to <60 ng/µL prior to ABC**[1]

Step	Instructions
8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

Tube	Component	Volume
Light blue	Annealing buffer	12.5 µL
Light green	Kinnex sequencing primer	12.5 µL
	Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[2,3]
- Recommend to **pool adapter-indexed 16S libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

PacBio

1 In the previous version of this procedure (103-238-800 Rev 03), Kinnex 16S RNA library is eluted in in **20 µL** EB and the DNA concentration must be less than 60 ng/µL to go into ABC.
2 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 7 in protocol) for performing sample setup ABC.
3 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (130 – 160 pM OPLC).

Revio system v13.3 PureTarget sequencing protocol updates

PureTarget sequencing protocol supports reduced DNA input requirements for PureTarget library preparation and includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Generating PureTarget repeat expansion panel libraries [103-329-400 Rev 03]

QC

1 Input DNA sample QC

PureTarget library construction

2 Dephosphorylation

3 Cas9-gRNA complex formation

4 Cas-9 digestion & cleanup

5 dA-tail

6 Adapter ligation & cleanup

7 Nuclease treatment

8 Sample pooling & cleanup

9 SMRT boost wash & cleanup

10 Final concentration

ABC

11 Annealing, binding & cleanup

The recommended mass of gDNA is **2 µg per sample** to ensure there are sufficient gene copies to load and maximize sequencing coverage. This protocol is suitable for 1–4 µg per sample. We recommend a minimum total DNA of 16 µg on the Sequel, Vega and Revio systems to yield a measurable library mass, and a maximum total DNA of 75 µg on the Sequel system, 100 µg on the Vega and non-SPRQ Revio system, and 50 µg on the Revio-SPRQ system, across all multiplexed samples.

System	Min gDNA input	Max gDNA input
Sequel II/e	16 µg	75 µg
Vega	16 µg	100 µg
Revio (non-SPRQ)	16 µg	100 µg
Revio (SPRQ)	16 µg	50 µg

- Added table to clarify per-SMRT Cell genomic DNA input requirements for each sequencing system[1]
  - Recommend **min. total input gDNA = 16 µg** per Revio SMRT Cell (across all multiplexed samples) to yield measurable library mass
  - Recommend **max. total input gDNA = 50 µg** for Revio-SPRQ chemistry (across all multiplexed samples)
- Protocol supports **1–4 µg** input gDNA per sample

No major changes to PureTarget library construction steps

✔	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.

- Updated section (Step 11) to include **ABC procedure and final loading dilution procedure for Revio SPRQ chemistry**
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

[1] Example input gDNA use cases for PureTarget: If user wants to run an 8-plex with Revio SPRQ chemistry, then min. input gDNA mass per sample is 2 µg (but using 2-4 µg per sample is still acceptable. If user wants to run a 48-plex with Revio SPRQ chemistry, then max. input gDNA mass per sample is 1 µg (since max. total input gDNA per Revio SPRQ SMRT Cell is 50 µg).

Revio system v13.3 SMRTbell prep kit 3.0 amplicon seq. protocol updates

SPK 3.0 multiplexed amplicon sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 [102-359-000 Rev 04]

QC

Multiplexed amplicon library construction

ABC

1. Input DNA sample QC
2. DNA repair & A-tailing
3. Adapter ligation & cleanup
4. Nuclease treatment & cleanup
5. Pooling & concentration (For adapter-indexed amplicons)
6. Annealing, binding & cleanup

No major changes to input amplicon DNA sample QC steps

PCR-indexed amplicon samples

Mean size	Minimum pooled amount per Revio SMRT Cell
<3 kb	300 ng
3–12 kb	500 ng
>12 kb	750 ng

Adapter-indexed amplicon samples

Mean size	Minimum per sample amount per Revio SMRT Cell
<3 kb	300 ng
3–12 kb	500 ng
>12 kb	750 ng

- Input DNA mass requirements for amplicon libraries remains the **same** for Revio SPRQ and non-SPRQ samples

5. Pooling & concentrating samples with indexed SMRTbell adapters

✓	Step	Instructions
	5.11	Remove the tube from the magnetic rack. **Immediately** add **26 µL** of **elution buffer** to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	5.17	If necessary, dilute 25 µl of SMRTbell library to the concentrations indicated below. Failure to normalize libraries or pools of libraries to the appropriate concentration prior to ABC may result in low sequencing yield.

SMRTbell library size	Concentration (ng/µl)
>10kb	<60 ng/µl
3 – 10 kb	<20 ng/µl
<3 kb	<10 ng/µl

✓	Step	Instructions
	6.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✓	Tube	Component	Volume
	Light blue	Annealing buffer 96	12.5 µL
	Light green	Standard sequencing primer 96	12.5 µL
		Total volume	**25 µL**

Minor updates to SMRTbell prep kit 3.0 amplicon library construction steps

- During pooling & concentration of adapter-indexed amplicon samples (**Step 5**), **elute pooled library in 26 µL EB (instead of 15 µL)**[1]
- **Added table to clarify recommendations for normalizing DNA concentrations prior to ABC depending on insert size range**

- New section (Step 6) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[2, 3]
- Recommend to **pool adapter-indexed HiFi libraries post-ABC**
- If samples are pooled prior to ABC or a custom volume is required, see protocol Appendix
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

1 In the previous version of this procedure (1102-359-000 Rev 03), pooled library is eluted in in 15 µL EB and the DNA concentration must be less than 60 ng/µL to go into ABC

2 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 6 in protocol) for performing sample setup ABC.

3 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (200 – 300 pM OPLC).

Revio system v13.3 HiFi plex prep kit 96 amplicon seq. protocol updates

HiFi plex prep kit 96 multiplexed amplicon sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing multiplexed whole genome and amplicon libraries using the HiFi plex prep kit 96 [103-418-800 Rev 03]

QC
- DNA sample QC

DNA shearing
- 1 Pipette DNA shearing **(skip for amplicon samples)**
- 2 Post-shearing cleanup

Multiplexed WGS library construction
- 3 DNA repair & A-tailing
- 4 Adapter ligation & pooling
- 5 Pool cleanup
- 6 Nuclease treatment (24-plex sample pool)
- 7 Final cleanup

ABC
- 8 Annealing, binding & cleanup

No major changes to input amplicon DNA sample QC steps

gDNA	Amplicons
50–300 ng per sample	20–200 ng per sample

- Required input DNA mass (**20 – 200 ng per sample**) for adapter-indexed amplicon libraries remains the **same** for Revio SPRQ and non-SPRQ samples

7. Pooling and SMRTbell cleanup bead purification

Pool the completed nuclease reactions based on the number of SMRTcells to be run. For example, if 96 samples will be sequenced on a single SMRTcell, pool four 24-sample nuclease reactions prior to SMRTbell cleanup.

Nuclease reactions pooled	Total volume	SMRTbell cleanup bead volume for 1.0x	SMRTbell cleanup bead volume for 1.3x	Elution volume
1	50 µl	50 µl	65 µl	25 µl
2	100 µl	100 µl	130 µl	25 µl
3	150 µl	150 µl	195 µl	25 µl
4	200 µl	200 µl	260 µl	25 µl

Minor updates to HiFi plex prep kit 96 amplicon library construction steps

- Added table to **clarify pooling options and SMRTbell cleanup bead volumes to use for post-nuclease cleanup step (Step 7)** [each nuclease reaction contains up to 24 adapter-indexed samples][1]

✔	Step	Instructions
	8.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer 96	12.5 µL
	Light green	Standard sequencing primer 96	12.5 µL
		Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[2,3]
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

1 In the previous version of this procedure (103-418-800 Rev 02), each nuclease reaction (containing up to 24 pooled samples) is purified **individually** instead of being pooled together.
2 To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 8 in protocol) for performing sample setup ABC.
3 After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (200 – 300 pM OPLC).

Revio system v13.3 AAV sequencing protocol updates

Adeno-associated virus (AAV) sequencing protocol includes updated sample setup (ABC) guidance for Revio SPRQ polymerase kit

Preparing multiplexed AAV SMRTbell libraries using SMRTbell prep kit 3.0 [102-126-400 Rev 04]

AAV DNA extraction & QC

1. DNase I treatment
2. AAV DNA extraction
3. Thermal annealing for ssAAV vectors

AAV library construction

4. Normalize DNA input & cleanup
5. Repair and A-tailing
6. Adapter ligation & cleanup
7. Nuclease treatment & cleanup
8. Pool libraries & concentrate

ABC

9. Annealing, binding & cleanup

No major changes to AAV DNA extraction & QC steps

Multiplex level	DNA Input
4-plex	250 ng per sample
8-plex	125 ng per sample
12-plex	83 ng per sample
16-plex	63 ng per sample
24-plex	42 ng per sample

- Use a combined total of 1 µg of AAV DNA per SMRT Cell
  - This is the combined total of samples that will be multiplexed
- Per sample amounts should be 1 µg/number of samples
- An example of per sample input amounts are shown in the table at left

No major changes to AAV library construction steps

✔	Step	Instructions
	9.1	Prepare the appropriate volume of master mix with 10% overage using the per reaction volumes listed below.

Annealing mix

✔	Tube	Component	Volume
	Light blue	Annealing buffer	12.5 µL
	Light green	Standard sequencing primer	12.5 µL
		Total volume	**25 µL**

- New section (Step 8) describes **ABC procedure for Revio SPRQ samples using static reagent & buffer volumes**[1,2]
- **Polymerase-bound complexes** can be stored at 4°C for 1 month or at -20°C for at least 6 months

PacBio

[1] To prepare Revio non-SPRQ libraries for sequencing, proceed to use SMRT Link Sample Setup module (instead of following Step 9 in protocol) for performing sample setup ABC.
[2] After ABC, proceed to the Loading Calculator in SMRT Link to prepare DNA internal control and final loading dilution (200 – 300 pM OPLC).

Nanobind HMW DNA extraction procedure for saliva samples

Procedure & checklist – Extracting HMW DNA from saliva using Nanobind kits (103-544-000)

Procedure & checklist describes the extraction of HMW DNA (50 – 300+ kb) from saliva collected with a Genotek Oragene DNA saliva collector (e.g., OG-500 or OG-600) using Nanobind kits.

Saliva sample input requirements

- Use 500 µL of input saliva collected and stabilized in a Oragene device (DNA Genotek)
- Perform pre-extraction QC using Qubit BR assay to verify saliva sample contains >2 µg of DNA in 500 µL for efficient extraction using Nanobind kits[1]
- Saliva collected in Oragene devices is stable at RT for up to 5 years (see DNA Genotek white paper)
- DNA yield for saliva samples extracted using Nanobind kits can vary from ~1 to ~45 µg depending on the donor

Collect saliva sample using Oragene device following DNA Genotek instructions

Use 500 µL input saliva stabilized in Oragene device buffer

Verify input DNA >2 µg in 500 µL saliva sample using Qubit BR assay[1]

Perform saliva HMW DNA extraction using Nanobind PanDNA kit or Nanobind CBB kit

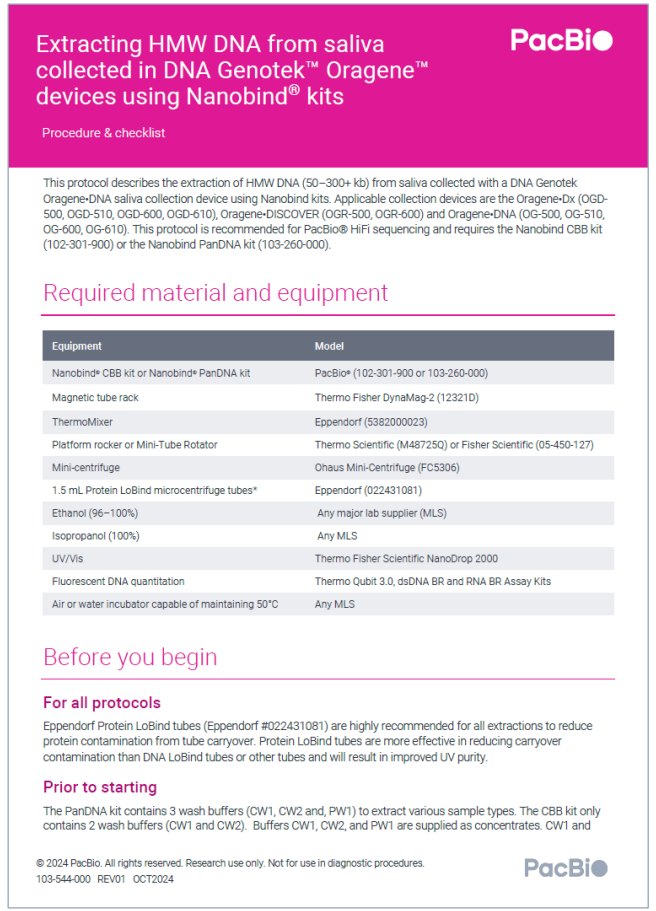
Step 1
Saliva collection
Pre-extraction QC

Step 2
Lyse white blood cells

Step 3
Bind

Step 4
Wash

Step 5
Elute



Extracting HMW DNA from saliva collected in DNA Genotek™ Oragene™ devices using Nanobind® kits

PacBio

Procedure & checklist

This protocol describes the extraction of HMW DNA (50–300+ kb) from saliva collected with a DNA Genotek Oragene•DNA saliva collection device using Nanobind kits. Applicable collection devices are the Oragene•Dx (OGD-500, OGD-510, OGD-600, OGD-610), Oragene•DISCOVER (OGR-500, OGR-600) and Oragene•DNA (OG-500, OG-510, OG-600, OG-610). This protocol is recommended for PacBio® HiFi sequencing and requires the Nanobind CBB kit (102-301-900) or the Nanobind PanDNA kit (103-260-000).

Required material and equipment

Equipment	Model
Nanobind® CBB kit or Nanobind® PanDNA kit	PacBio® (102-301-900 or 103-260-000)
Magnetic tube rack	Thermo Fisher DynaMag-2 (12321D)
ThermoMixer	Eppendorf (5382000023)
Platform rocker or Mini-Tube Rotator	Thermo Scientific (M48725Q) or Fisher Scientific (05-450-127)
Mini-centrifuge	Ohaus Mini-Centrifuge (FC5306)
1.5 mL Protein LoBind microcentrifuge tubes*	Eppendorf (022431081)
Ethanol (96–100%)	Any major lab supplier (MLS)
Isopropanol (100%)	Any MLS
UV/Vis	Thermo Fisher Scientific NanoDrop 2000
Fluorescent DNA quantitation	Thermo Qubit 3.0, dsDNA BR and RNA BR Assay Kits
Air or water incubator capable of maintaining 50°C	Any MLS

Before you begin

For all protocols

Eppendorf Protein LoBind tubes (Eppendorf #022431081) are highly recommended for all extractions to reduce protein contamination from tube carryover. Protein LoBind tubes are more effective in reducing carryover contamination than DNA LoBind tubes or other tubes and will result in improved UV purity.

Prior to starting

The PanDNA kit contains 3 wash buffers (CW1, CW2 and, PW1) to extract various sample types. The CBB kit only contains 2 wash buffers (CW1 and CW2). Buffers CW1, CW2, and PW1 are supplied as concentrates. CW1 and

103-544-000 REV01 OCT2024

PacBio

PacBio Documentation (103-544-000)

[1] If pre-extraction DNA concentration measurement is <4 ng/µL (<2 µg in 500 µL), Nanobind extraction recovery may be low and we recommend recollecting the saliva sample for extraction.

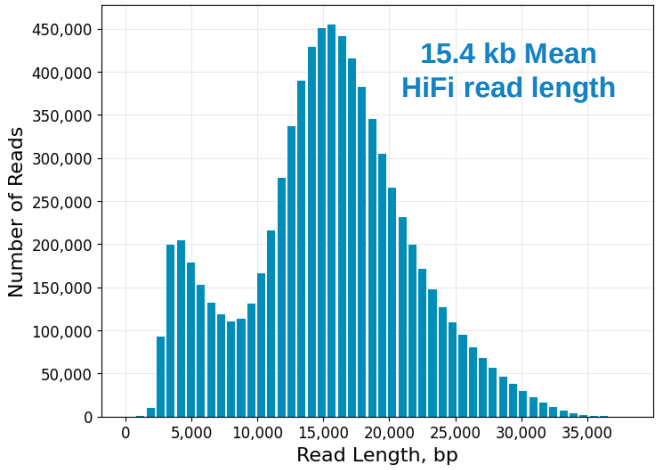
Revio system v13.3 example sequencing performance

Revio SPRQ whole genome sequencing performance

Human WGS

Example human WGS variant & methylation calling performance data for HG002 using 500 ng of input DNA

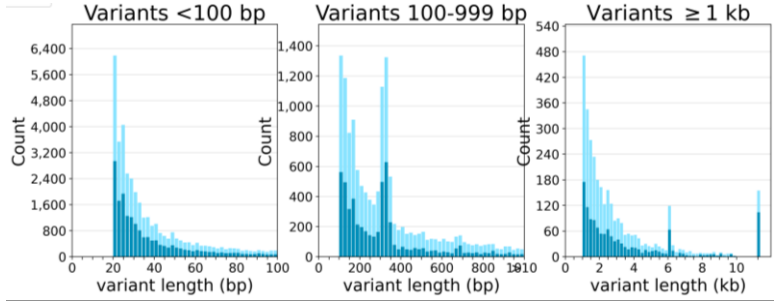
Sequencing metrics

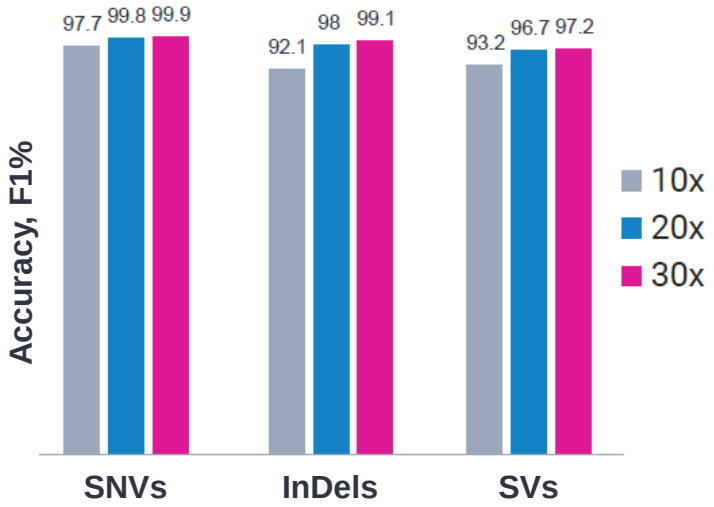


HiFi Reads	8.4 M
HiFi Base Yield	119.7 Gb
Mean HiFi Read Length	15.4 kb
Median HiFi Read Quality	Q33
HiFi Read Mean # of Passes	10

For human HG002 WGS libraries run with Revio SPRQ chemistry, per-SMRT Cell HiFi read counts were typically >7 Million depending on final library insert size and *P1* loading[1]

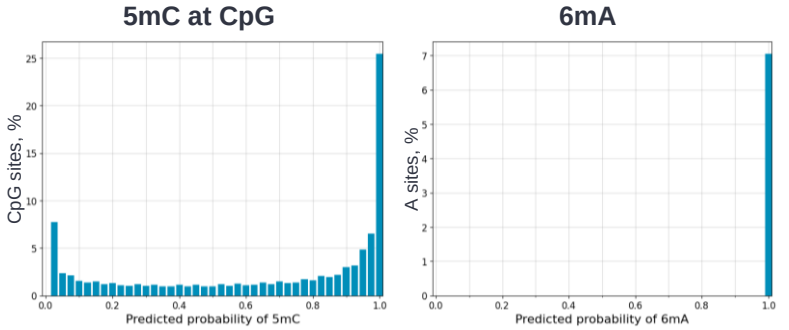
Variant calling performance[2]



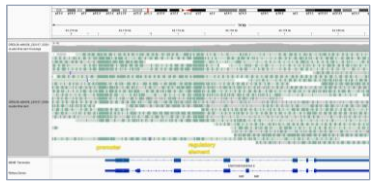


Methylation calling performance

Modificati...	Motif	Scored sites	Modified sites (Pr > 0.5)
5mC	CpG	97.5%	62.3%
6mA	A	7.0%	7.0%



	5mC (CpG)
Sensitivity	93%
Specificity	93%



5mC data show a strongly bimodal profile indicating increased confidence of true positive and true negative 5mC calls

Revio on-instrument calling has high accuracy for 6mA, equivalent to Fibertools for Fiber-seq assays.[3]

PacBio

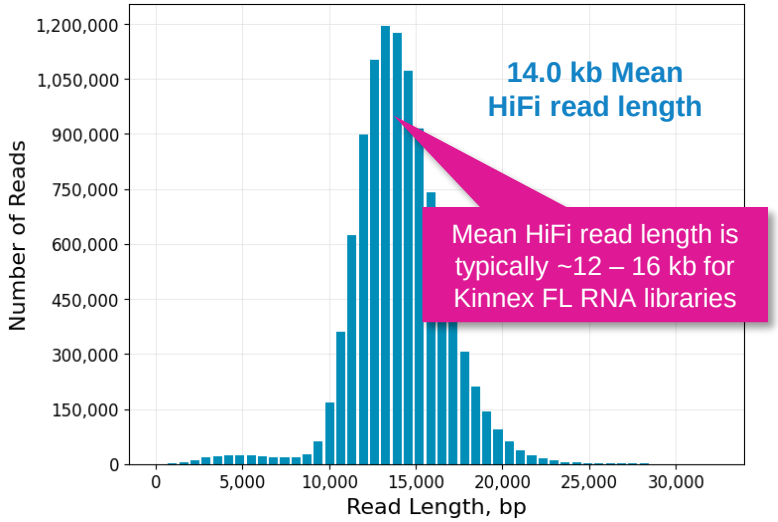
1 Example sequencing metrics shown for human HG002 sample that was loaded at 300 pM OPLC and achieved *P1* = 69% using 500 ng of input gDNA.
2 F1% accuracy score data shown are for a single Revio SMRT Cell for HG002/GM24385 sequenced with Revio SPRQ chemistry.
3 Revio on-instrument 6mA caller has > 90% sensitivity for 6mA in Fiber-seq and < 5 false positive calls per 1,000 A bases in reads.

Revio SPRQ RNA sequencing performance

KINNEX

Example Kinnex full-length RNA sequencing performance for Universal Human Reference RNA sample (6-plex)

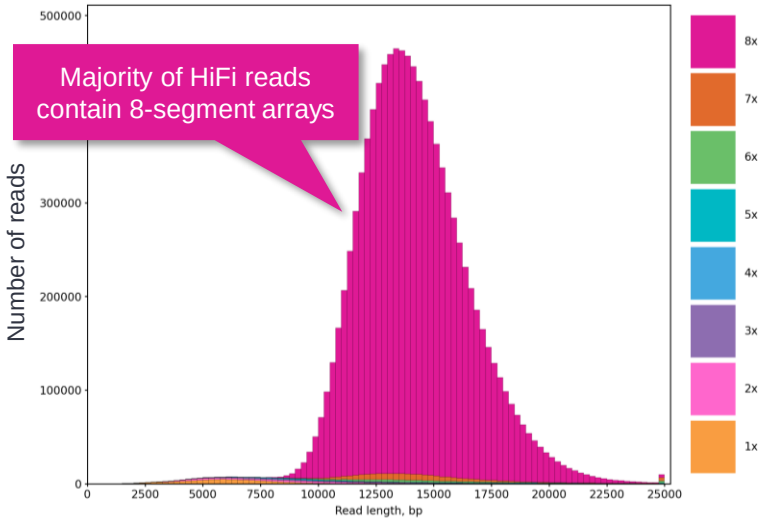
Sequencing metrics



HiFi Reads	10.4 M
HiFi Base Yield	146.76 Gb
Mean HiFi Read Length	14.0 kb
Median HiFi Read Quality	Q32
HiFi Read Mean # of Passes	7

For UHRR Kinnex full-length RNA libraries, per-Revio SMRT Cell HiFi read counts were typically >7 Million depending on final library insert size and *P1* loading.[1]

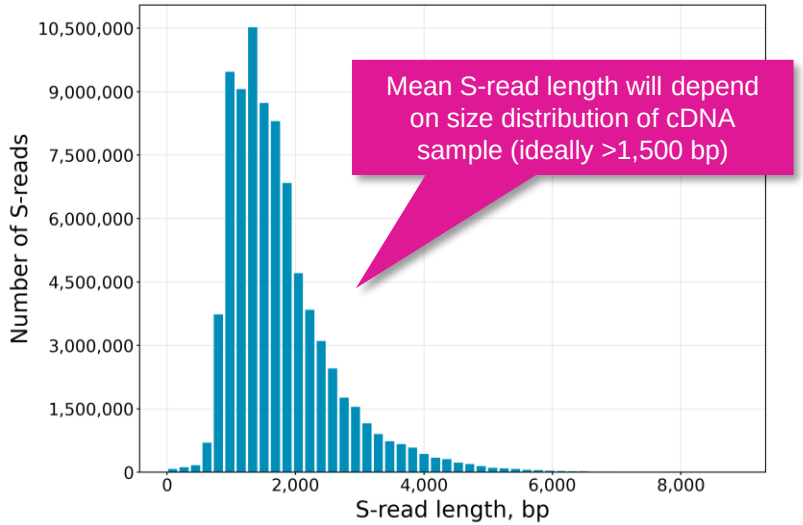
Read segmentation



Input HiFi Reads	10,448,251
Percent of reads with full arrays	94.93%
Mean array size (concentration factor)	7.79

Histogram distribution of number of HiFi reads by read length, in bp. For UHRR Kinnex libraries, % of reads with full arrays is ideally >80% and mean array size is ideally >7.0 segments.

Length of S-reads



Mean length of S-reads	1,758 bp
Segmented reads (S-reads)	81,377,012

Histogram distribution of the number of S-reads by HiFi read length, in base pairs. Mean S-read length will depend on size distribution of cDNA sample (ideally >1,500 bp).

PacBio

[1] Example sequencing metrics shown for UHRR sample that was loaded at 160 pM OPLC and achieved *P1* = 80% using 300 ng of input cDNA per sample.

Revio SPRQ RNA sequencing performance

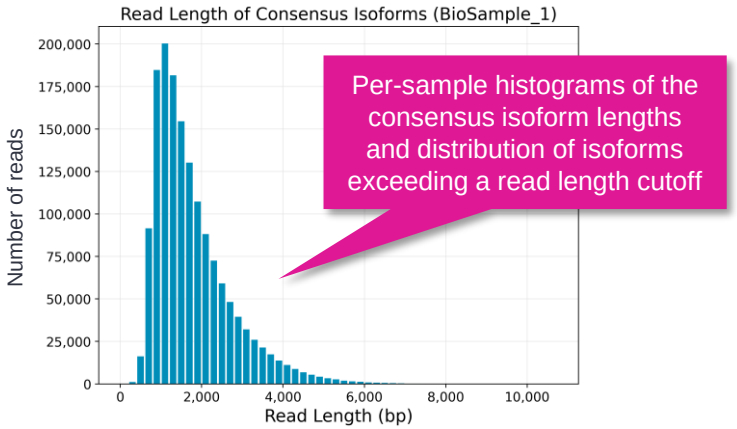
KINNEX

Example Kinnex full-length RNA sequencing performance for Universal Human Reference RNA sample (6-plex)

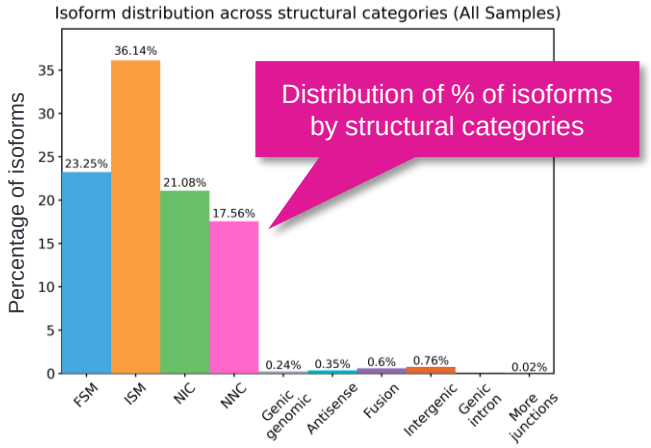
Transcript clustering summary metrics

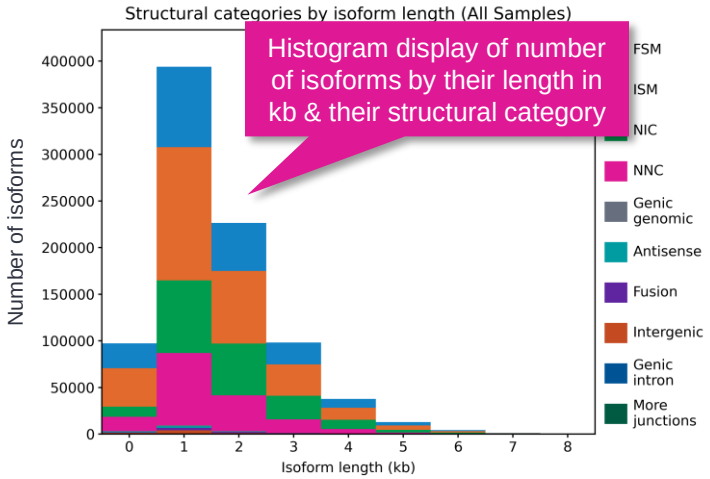
Sample Name	Number of High-Quality Isoforms
BioSample_1	1,537,224
BioSample_2	1,555,337
BioSample_3	1,452,679
BioSample_4	1,455,390
BioSample_5	1,447,673
BioSample_6	1,527,724
All Samples	2,941,510

Number of consensus isoforms that have an estimated accuracy above specified threshold

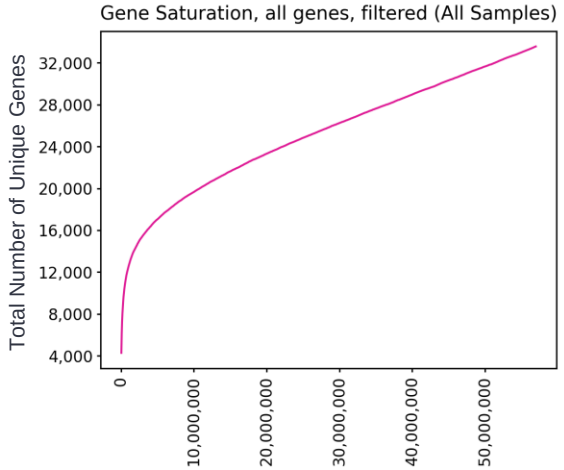


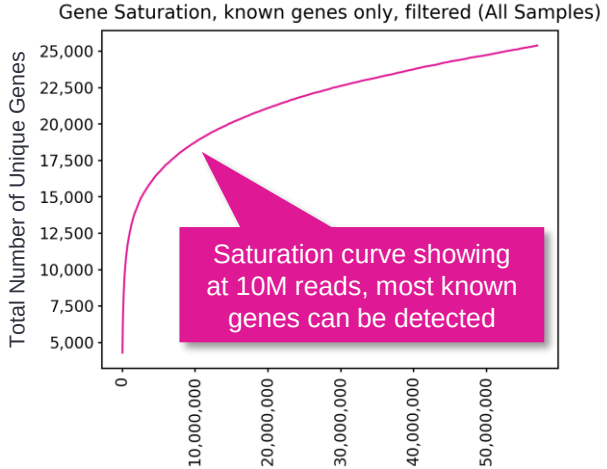
Transcript classification plots





Gene saturation plots[1]





PacBio

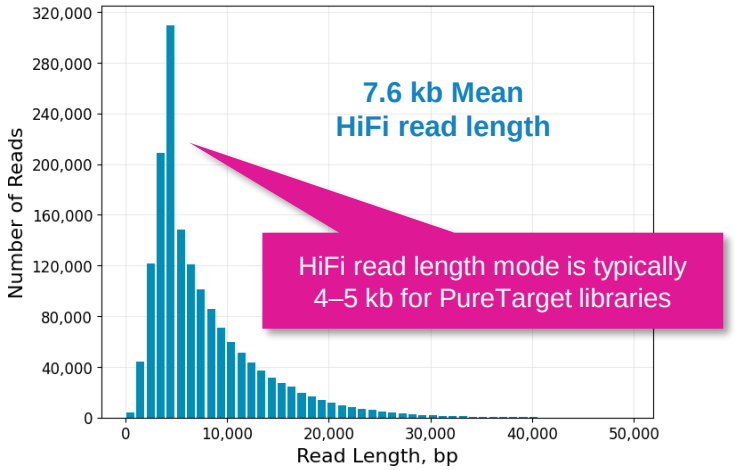
[1] Gene Saturation, all genes, filtered: Saturation plot showing the level of gene saturation for **all genes**, after filtering out reads based on the SQANTI transcript filtering criteria. Gene Saturation, known genes only, filtered: Saturation plot showing the level of gene saturation, for unique **known genes only** (genes annotated in the reference annotation) per cell, after filtering out reads based on the SQANTI transcript filtering criteria.

Revio SPRQ targeted sequencing performance

PureTarget™

Example PureTarget sequencing performance for Coriell Institute human gDNA sample (24-plex)

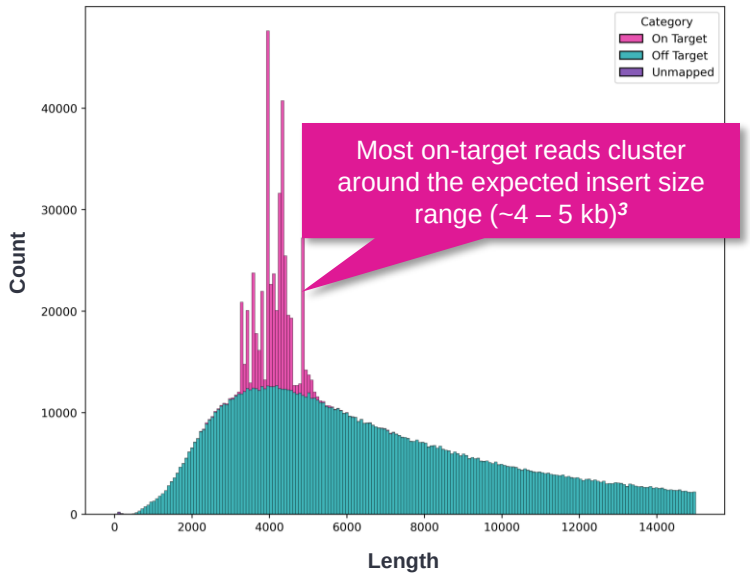
Sequencing metrics



HiFi Reads	1.6 M
HiFi Base Yield	12.2 Gb
Mean HiFi Read Length	7.6 kb
Median HiFi Read Quality	Q39
HiFi Read Mean # of Passes	22

For 24-plex repeat expansion panel libraries,[1] per-Revio SMRT Cell HiFi read counts were typically >1 Million depending on final library insert size and *P1* loading[2]

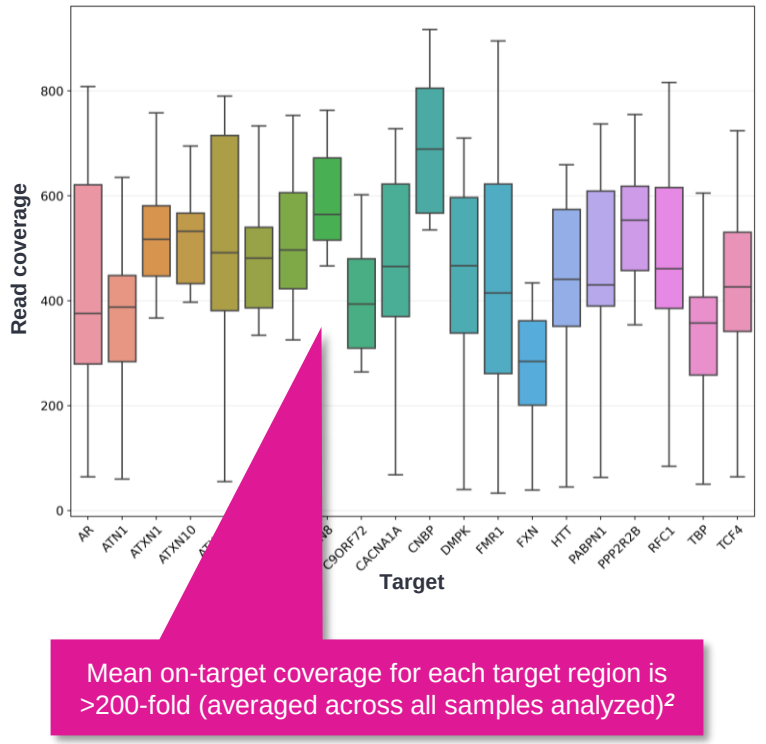
Read categories



Total Reads	1,631,763
Sample Count	24
Target Regions	20

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

Target coverage



Box plot for each target region of mean coverage across all samples analyzed → Can quickly compare coverage across all target regions and identify targets with low/high coverage

PacBio

1 For PureTarget library construction, 1000 ng of input gDNA was used per sample (except for two samples that used 320 ng each).

2 Example sequencing metrics shown for Coriell Institute human samples that achieved *P1* = 31%. **Note:** When evaluating PureTarget runs, it is useful to examine the secondary analysis results (e.g., on-target coverage) since primary sequencing metrics like *P1* (%) are mostly dominated by 'background' non-targeted reads..

3 PureTarget library inserts with expanded alleles may generate on-target reads longer than ~4 – 5 kb.

Revio system v13.3 with SPRQ chemistry example datasets

Example Revio SPRQ datasets are available for WGS, PureTarget and Kinnex full-length RNA

Application	Dataset	Data type	PacBio system
Whole genome sequencing			
Variant detection, assembly, epigenetics	Homo sapiens - GIAB trio HG002-4	HiFi long read	Revio system – SPRQ chemistry
Tumor/normal	COLO829 melanoma	HiFi long read	Revio system – SPRQ chemistry
Whole genome sequencing - Fiber-Seq chromatin assay	Homo sapiens - HG002	HiFi long read	Revio system – SPRQ chemistry
Targeted sequencing			
PureTarget	Repeat expansion panel - Coriell samples	HiFi long read	Revio system – SPRQ chemistry
RNA sequencing			
Kinnex full-length RNA	Homo sapiens - UHRR	HiFi long read	Revio system – SPRQ chemistry

Revio system v13.3 summary

Revio system v13.3 + SPRQ chemistry key benefits summary

Revio system with SPRQ chemistry reduces sequencing costs and enables more sample types & capabilities

4× lower DNA input

500 ng DNA input per Revio SMRT Cell

Compatible with existing library prep workflows

→ Unlock more sample types (e.g., tumor, saliva, small plant/animal, etc.)

33% increased HiFi yield

120 Gb per Revio SMRT Cell

Two 20× human genomes per Revio SMRT Cell

→ 2,500 HiFi genomes per year

Expanded epigenetics capabilities

10% increase in 5mC calling accuracy

On-instrument 6mA caller compatible with Fiber-seq assays

→ More accurate & streamlined multi-omics studies

Revio v13.3 with SPRQ chemistry extends the core capabilities of the Revio system

Revio system v13.3 specifications

Library	Run time[1]	Q30+ bases	HiFi yield per SMRT Cell[2]	Methylation
1–5 kb	12 hours	95%	6 – 8 Million reads	5mC at CpG sites and 6mA for native DNA
5–10 kb	24 hours	90%	35 – 70 Gb	
10–15 kb			70 – 100 Gb	
15–20 kb			100 – 120 Gb	
20–25 kb	30 hours	85%	100 – 120 Gb	

1 Run time refers to the data collection step, which determines the time between processing SMRT Cells.

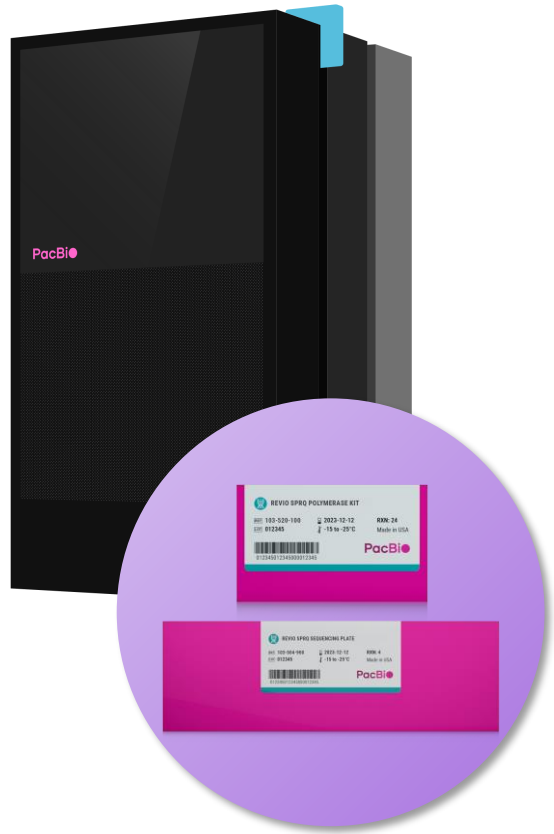
2 HiFi yield is dependent on library quality and sequencing preparation procedures. Specified yield is based on high-quality samples prepared following best practices.

Key applications and sample throughput (Revio system v13.3)

Library	Sample	Expected coverage[3]	Samples Per Revio SMRT Cell	Samples per year[4]
1–5 kb	Amplicon	50×	>1,000	>2.5M
5–10 kb	PureTarget repeat expansion panel	200×	48	60,000
5–10 kb	Microbial genome	30×	384	480,000
15–20 kb	Human genome	20×	2	2,500
15–20 kb	Human methylation profiling	5×	8	10,000
15–20 kb	Transcriptome with Kinnex full-length RNA kit	10M reads	6	7,500

3 Expected coverages are estimates.

4 Annual throughput is estimated and based on 2,500 Revio SMRT Cells for 12 hour runs; 1,250 Revio SMRT Cells for 24 hr runs; and 1,050 for 30 hour runs.



Revio system v13.3
+
SPRQ chemistry

See ***Revio system specification sheet*** (102-326-552) for the latest Revio system performance specifications.

Revio system v13.3 with SPRQ chemistry enables HiFi sequencing at scale

See ***What can you do with one SMRT Cell*** (102-326-578)

Vega system

Revio system with SPRQ chemistry

Application	Samples per run		
	1 SMRT Cell	1 SMRT Cell	4 SMRT Cells
Whole genome sequencing			
Human genome (20× coverage)	1	2	8
Human methylation profiling (5× coverage)	4	8	32
De novo assembly (1 Gb genome)	2	4	16
Microbial de novo assembly (1 Gb total sum of genomes)	384	384	1,536
Targeted panels			
Amplicon sequencing	>1,000	>1,000	>1,000
Target enrichment			
20 Mb panel	12	16	64
2 Mb panel	72	96	384
100 kb panel	288	384	1,536
PureTarget repeat expansions panel	48	48	192
RNA sequencing			
Kinnex single-cell RNA sequencing	1 (3,000 – 6,000 cells)	1 (6,000 – 10,000 cells)	4 (6,000 – 10,000 cells)
Kinnex full-length RNA sequencing			
5M reads	6	12	48
10M reads	3	6	24
Microbial			
Shotgun metagenomic profiling	64 communities	128 communities	512 communities
Shotgun metagenomic assembly	8 communities	16 communities	64 communities
Kinnex 16S rRNA	1,024 communities	1,536 communities	6,144 communities

All sample throughputs are estimates for either the Vega system with 1 SMRT Cell or the Revio system using SPRQ chemistry with both 1 or 4 SMRT Cells. Coverage may vary based on sample quality, library quality, and fragment lengths. Currently available SMRTbell® adapter index plates 96A-96D contain a total of 384 SMRTbell barcoded adapters. Microbial de novo assembly assumes microbes with 2 Gb of total genome size at 30x per sample. Single-cell transcriptomics assumes ≥80 million reads per library on the Revio system and ~50-60 million reads per library on the Vega system. Full-length RNA sequencing assumes a total of 60M reads for Revio SPRQ and 30M reads for Vega, regardless of plexity. Amplicon sequencing assumes a 12-hour movie time for 1–5 kb, 24-hour movie time for 5+ kb, and >50× per sample. Target enrichment assumes >50× per sample

PacBio

Technical documentation & applications support resources

Revio system and SMRT Link documentation

Revio system documentation

- Revio system Instrument Control Software release notes (103-593-500)
- Revio system v13.3 operations guide (102-962-600)
- Revio system specifications sheet (102-326-522)

SMRT Link & other data analysis documentation

- Application brief – SMRT Link (102-326-628)
- Brief primer and lexicon for PacBio SMRT sequencing webpage (v13.1)
- PacBio bioinformatics file formats documentation webpage (v13.1)
- SMRT Link v25.1 release notes (103-592-800)
- SMRT Link v25.1 software installation guide (103-566-000)
- SMRT Link v25.1 user guide (103-566-100)
- SMRT Link v25.1 web services API use cases [Link]
- SMRT Tools v25.1 reference guide [Link]

DNA sample extraction documentation

Brochures

- Brochure – Nanobind high-throughput HMW DNA extraction (102-326-565)
- Brochure – Nanobind PanDNA kit (102-326-604)

Technical notes

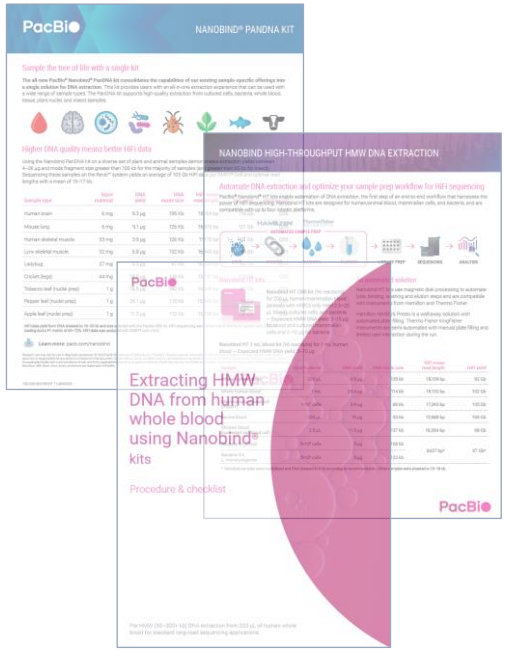
- Technical note – High-throughput DNA extraction (102-326-611)
- Technical note – Insect DNA extraction (102-326-612)
- Technical note – Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (102-193-651)
- Technical note – Sample preparation for PacBio HiFi sequencing from human whole blood (102-326-500)

Nanobind kit protocols and Guides & overviews

- Guide & overview – Nanobind CBB kit (102-572-200)
- Guide & overview – Nanobind PanDNA kit (103-394-800)
- Nanobind Procedures & checklists – see PacBio Documentation
- Overview – Nanobind CBB HMW DNA extraction protocols (103-515-700)
- Overview – Nanobind HT HMW DNA extraction robotic procedures (103-032-000)
- Overview – Nanobind PanDNA HMW DNA extraction protocols (103-510-000)
- Technical overview – HMW DNA sample preparation for PacBio long-read sequencing using Nanobind PanDNA and SRE kits (103-401-100)

Nanobind high-throughput (HT) automation kit protocols and Guides & overviews

- Guide & overview – Nanobind HT kits (103-028-100)
- Nanobind HT Procedures & checklists – see PacBio Documentation
- Technical overview – Automated high-throughput HMW DNA extraction for PacBio long-read sequencing using Nanobind HT kits (103-401-700)



PacBio

[1] Refer to PacBio Documentation website for the latest Nanobind HMW DNA extraction procedures.

SMRTbell library preparation documentation & other resources

SMRTbell library preparation literature

- Overview – HiFi application options (101-851-300)
- Procedure & checklist – Generating PureTarget repeat expansion panel libraries (103-329-400)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex single-cell RNA kit (103-254-300)
- Procedure & checklist – Preparing Kinnex libraries using Kinnex full-length RNA kit (103-238-700)
- Procedure & checklist – Preparing Kinnex libraries from 16s rRNA amplicons (103-238-800)
- Procedure & checklist – Preparing multiplexed AAV SMRTbell libraries using SMRTbell prep kit 3.0 (102-126-400)
- Procedure & checklist – Preparing multiplexed amplicon libraries using PacBio barcoded M13 primers and SMRTbell prep kit 3.0 (101-921-300)
- Procedure & checklist – Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 (102-359-000)
- Procedure & checklist – Preparing whole genome and metagenome sequencing libraries using SMRTbell prep kit 3.0 (102-166-600)

Hybrid capture library preparation literature

- Twist protocol – Long read library preparation and standard hyb v2 enrichment (DOC-001320)

Revio system applications support documentation

Application notes & best practices guides

Whole genome sequencing applications

- Application brief – Whole genome sequencing for de novo assembly – Best Practices (102-193-627)
- Application brief – Variant detection using whole genome sequencing with HiFi reads – Best Practices (102-193-604)
- Application brief – Microbial whole genome sequencing – Best Practices (102-193-601)

Viral sequencing applications

- Application brief – AAV sequencing – Best Practices (102-193-502)
- Application brief – Highly-accurate HiFi reads for AAV-research (102-326-594)

RNA sequencing applications

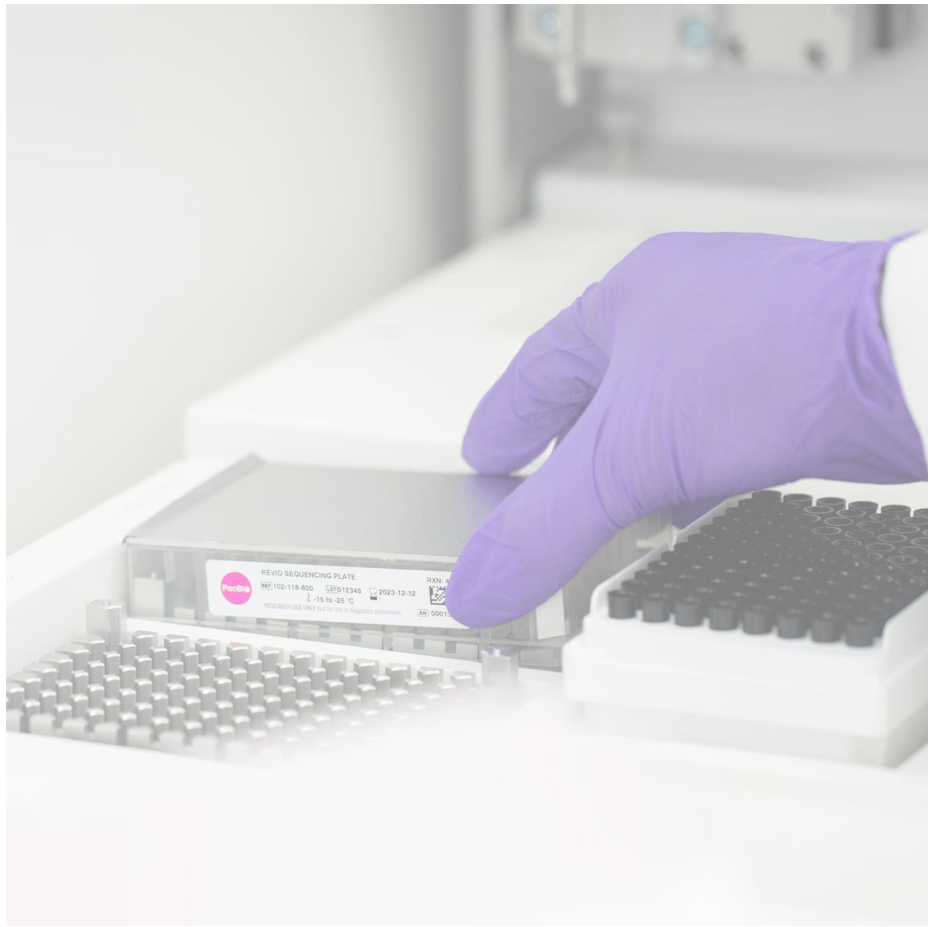
- Application note – Kinnex full-length RNA kit for isoform sequencing (102-326-591)
- Application note – Kinnex single-cell RNA kit for single-cell isoform sequencing (102-326-549)

Metagenomics applications

- Application note – Kinnex 16S rRNA kit for full-length 16S sequencing (102-326-601)
- Application brief – Metagenomic sequencing with HiFi reads – Best Practices (102-193-684)

Targeted sequencing applications

- Application brief – HiFi target enrichment – Best practices (102-326-515)
- Application brief – Targeted sequencing for amplicons – Best Practices (102-193-603)



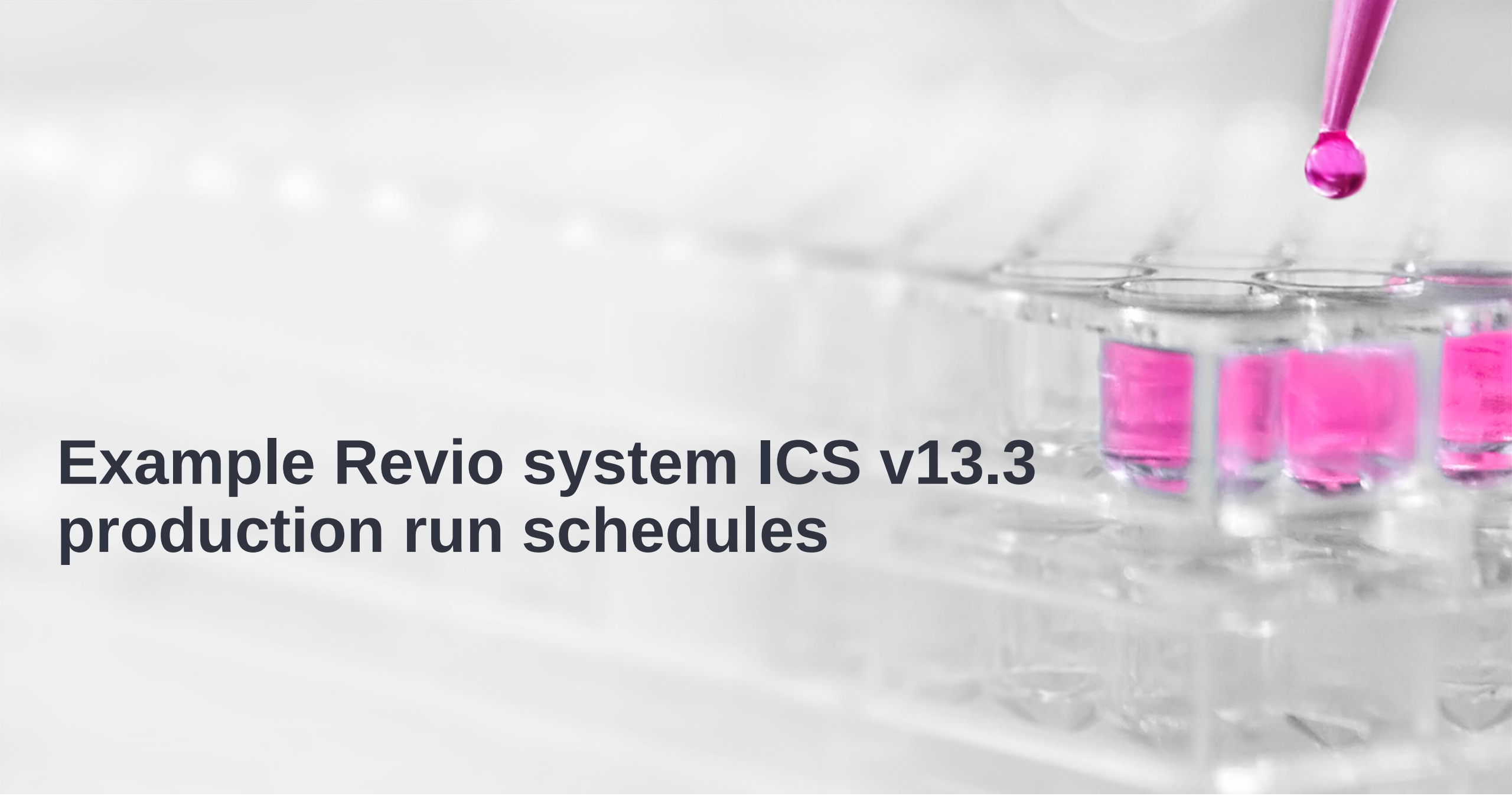
Revio system applications support documentation (cont.)

Application technical overviews

- Technical overview – Adeno-associated virus (AAV) library preparation using SMRTbell prep kit 3.0 (102-390-400)
- Technical overview – Kinnex kits for single-cell RNA and full-length RNA and 16S rRNA sequencing (103-343-700)
- Technical overview – Kinnex library preparation for full-length 16S rRNA sequencing (103-344-800)
- Technical overview – Kinnex library preparation using Kinnex full-length RNA kit (103-344-700)
- Technical overview – Kinnex library preparation using Kinnex single-cell RNA kit (103-344-600)
- Technical overview – Multiplexed amplicon library preparation using SMRTbell prep kit 3.0 (102-395-900)
- Technical overview – PureTarget repeat expansion panel library preparation using PureTarget kit (103-418-100)
- Technical overview – Whole genome and metagenome library preparation using SMRTbell prep kit 3.0 (102-390-900)

Appendix

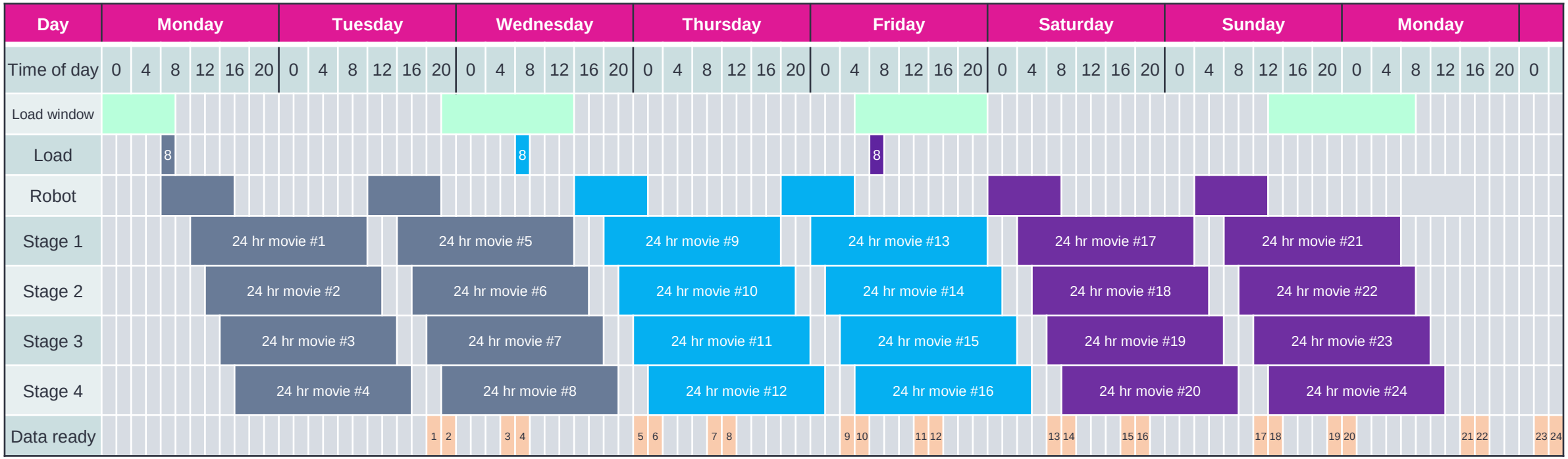
Example Revio system ICS v13.3 production run schedules



Revio high-utilization schedule: 24 hr movies, 8 SMRT Cells per run

24 SMRT Cells / week

Load same time of day on
Mon, Wed, Fri (3 touch points)

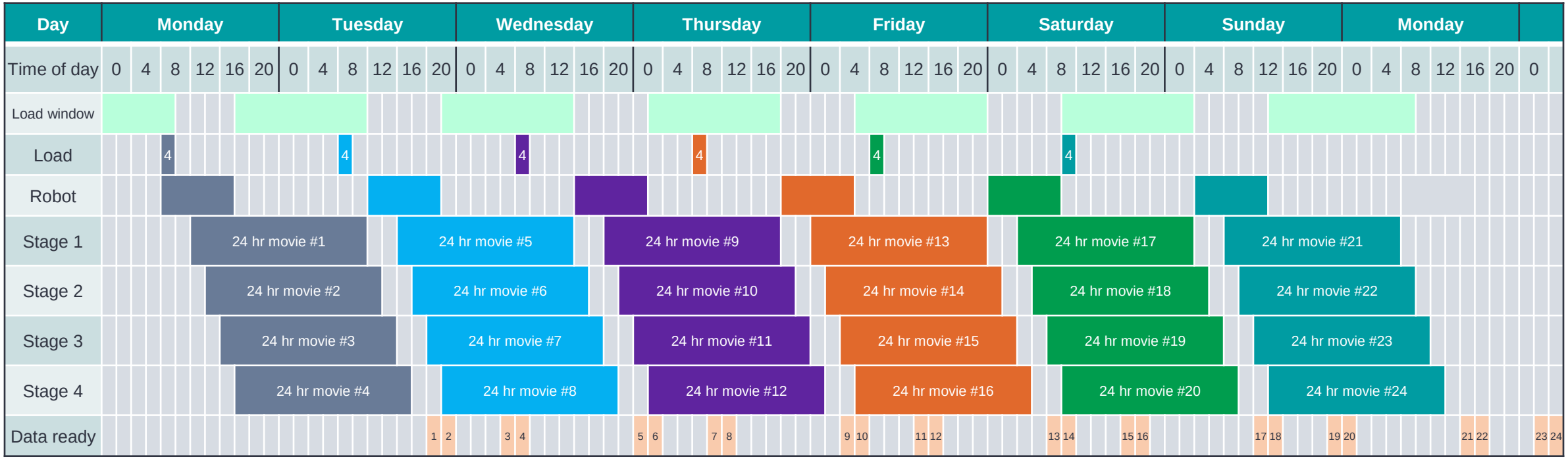


Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 576 hours of automated sequencing runtime per week (144 hours × 4 stages) and 3 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

Revio high-utilization schedule: 24 hr movies, 4 SMRT Cells per run

24 SMRT Cells / week

Load same time of day on Mon-Sat (6 touch points)

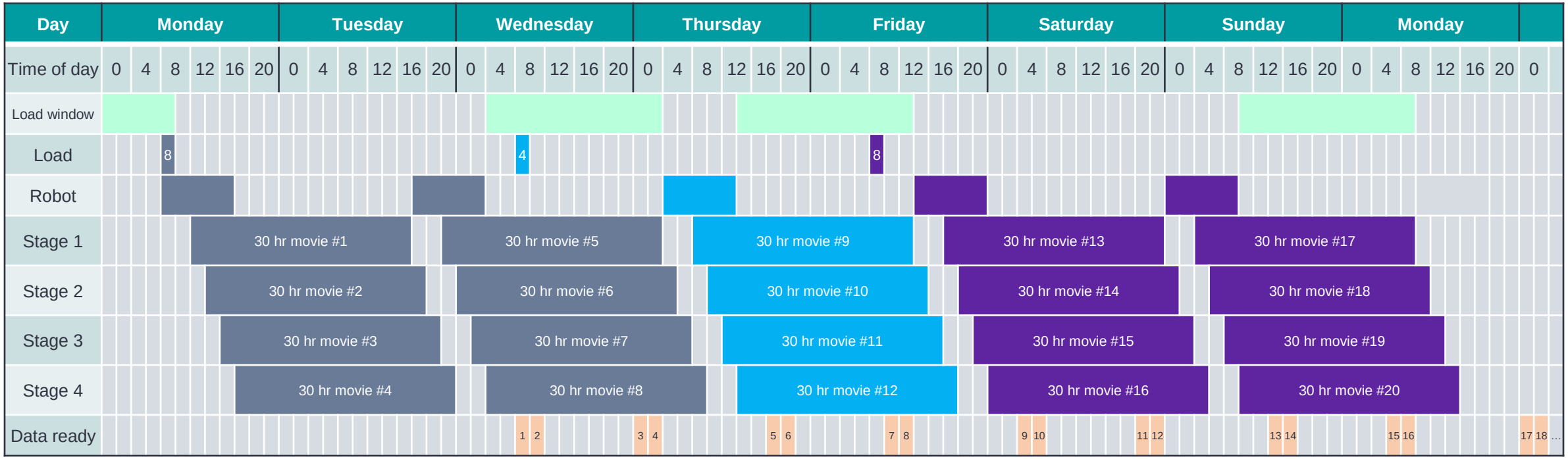


Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 576 hours of automated sequencing runtime per week (144 hours × 4 stages) and 6 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

Revio high-utilization schedule: 30 hr movies, 8+4+8 SMRT Cells per run

20 SMRT Cells / week

Load same time of day on
Mon, Wed, Fri (3 touch points)

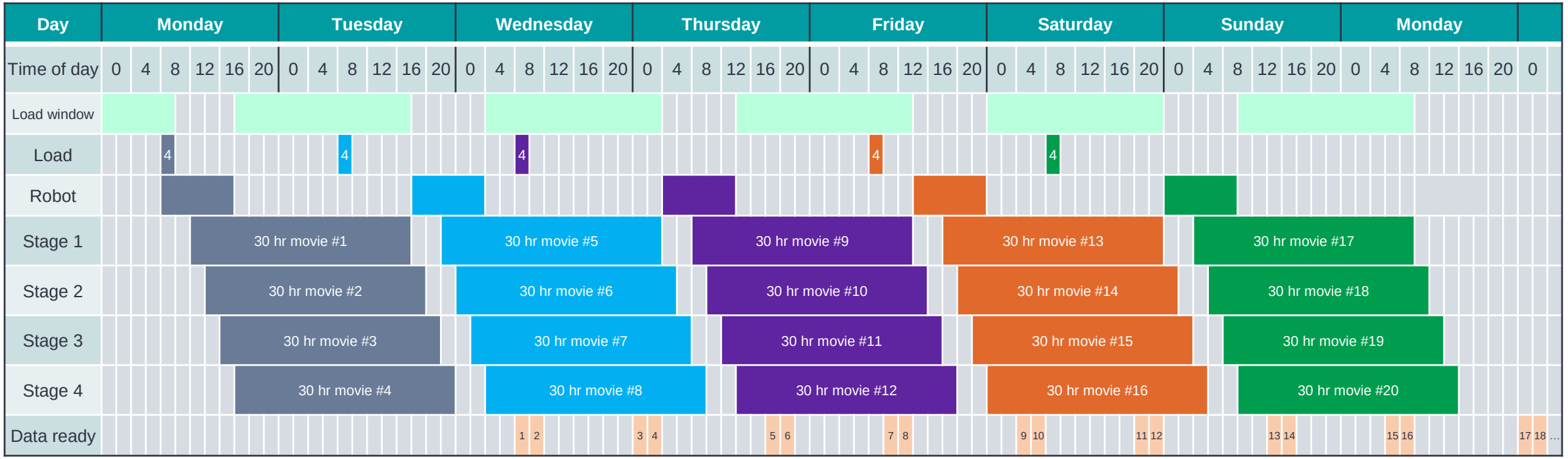


Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 600 hours of automated sequencing runtime per week (150 hours × 4 stages) and 3 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

Revio high-utilization schedule: 30 hr movies, 4 SMRT Cells per run

20 SMRT Cells / week

Load same time of day on
Mon, Tue, Wed, Fri, Sat (5 touch points)

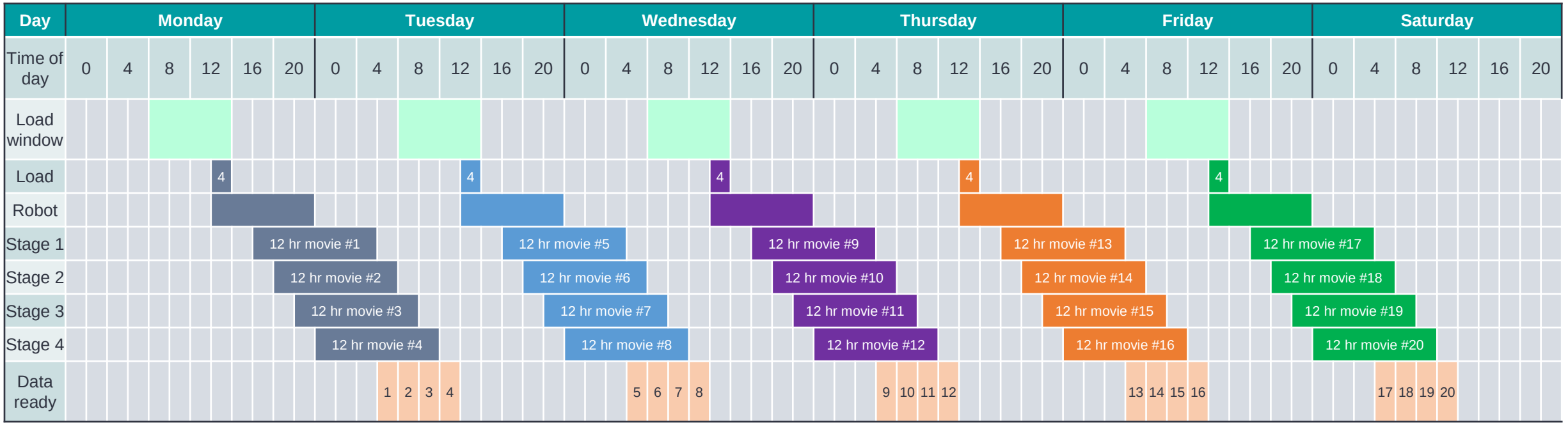


Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 600 hours of automated sequencing runtime per week (150 hours × 4 stages) and 5 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

Revio high-utilization schedule: 12 hr movies, 4 Cells per run

20 SMRT Cells / week

Load same time of day on Mon-Sat (5 touch points)

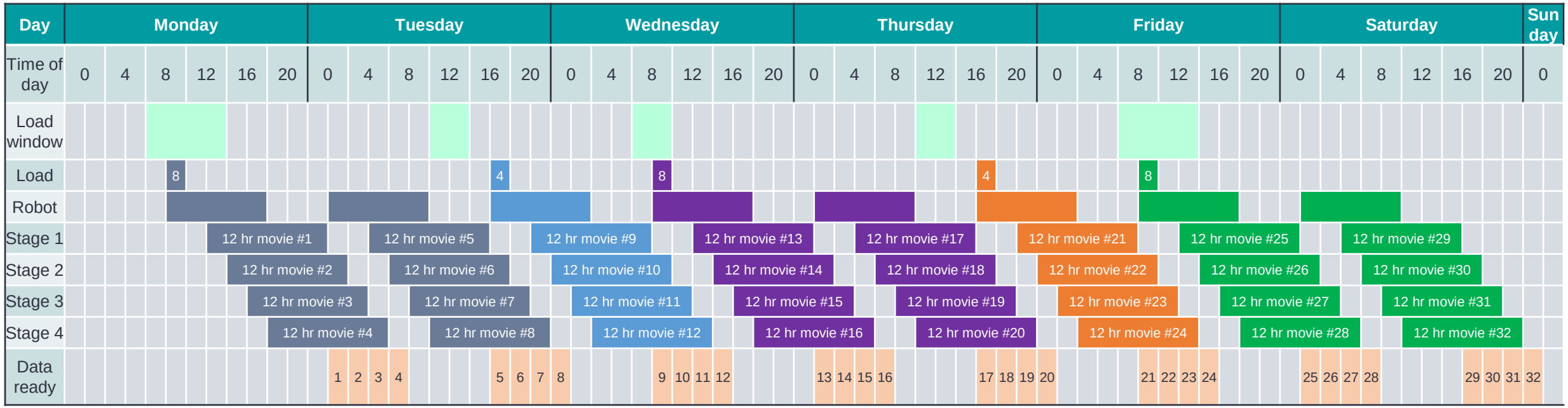


Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 240 hours of automated sequencing runtime per week (60 hours × 4 stages) and 5 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

Revio high-utilization schedule: 12 hr movies, 8+4+8+4+8 Cells per run

32 SMRT Cells / week

Load before 12pm on 8-cell run days
Load between 3pm-5pm on 4-cell run days
Mon-Sat (5 touch points)



Example high-throughput Revio run schedule uses default run conditions (including adaptive loading) and involves 384 hours of automated sequencing runtime per week (96 hours × 4 stages) and 5 manual touchpoints to pre-load sequencing consumables onto instrument work deck for continuous operation.

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