PACIFIC BIOSCIENCES®

Technical Overview: Low DNA Input Library Preparation Using SMRTbell Express Template Prep Kit 2.0

Sequel System ICS v8.0 / Sequel Chemistry 3.0 / SMRT Link v9.0
Sequel II System ICS v9.0 / Sequel II Chemistry 2.0 / SMRT Link v9.0
Sequel IIe System ICS v10.0 / Sequel II Chemistry 2.0 / SMRT Link v10.0

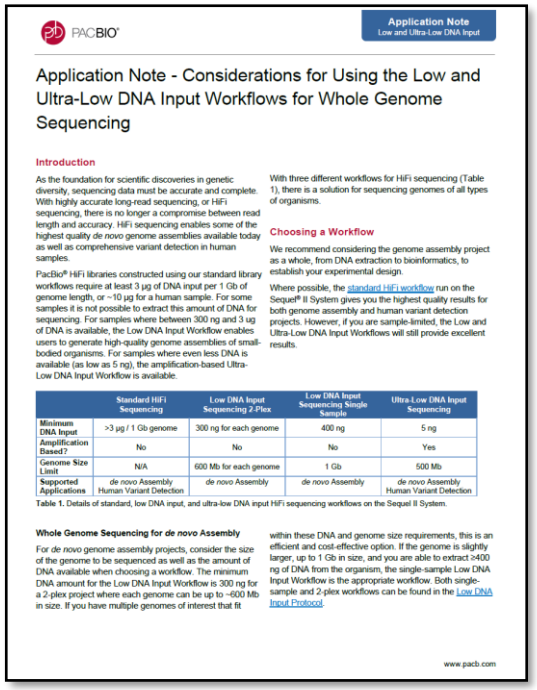
PN 101-781-000 Ver 2021-02-01-A (February 2021)

Low DNA Input Library Preparation Using SMRTbell Express Template Prep Kit 2.0

1. Low DNA Input Workflow Overview
2. Low DNA Input Sample QC Requirements
3. Low DNA Input Library Sample Preparation Workflow Details
4. Low DNA Input Library Sequencing Workflow Details
5. HiFi Sequencing Data Analysis Recommendations for *De Novo* Assembly Applications
6. Low DNA Input Library Example Performance Data
7. Technical Documentation & Applications Support Resources
8. Appendix: General Recommendations for High-Molecular Weight gDNA QC and Handling for SMRTbell Library Construction

LOW DNA INPUT SEQUENCING: HOW TO GET STARTED

Low- / Ultra-Low DNA Input Application Note → **Application-Specific Procedure & Checklist** → **Application Consumable Bundle Purchasing Guide** → **Library Construction, Sequencing & Analysis**



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Application Note
Low and Ultra-Low DNA Input

Application Note - Considerations for Using the Low and Ultra-Low DNA Input Workflows for Whole Genome Sequencing

Introduction

As the foundation for scientific discoveries in genetic diversity, sequencing data must be accurate and complete. With highly accurate long-read sequencing, or HiFi sequencing, there is no longer a compromise between read length and accuracy. HiFi sequencing enables some of the highest quality *de novo* genome assemblies available today as well as comprehensive variant detection in human samples.

PacBio® HiFi libraries constructed using our standard library workflows require at least 3 µg of DNA input per 1 Gb of genome length, or ~10 µg for a human sample. For some samples it is not possible to extract this amount of DNA for sequencing. For samples where between 300 ng and 3 ug of DNA is available, the Low DNA Input Workflow enables users to generate high-quality genome assemblies of small-bodied organisms. For samples where even less DNA is available (as low as 5 ng), the amplification-based Ultra-Low DNA Input Workflow is available.

With three different workflows for HiFi sequencing (Table 1), there is a solution for sequencing genomes of all types of organisms.

Choosing a Workflow

We recommend considering the genome assembly project as a whole, from DNA extraction to bioinformatics, to establish your experimental design.

Where possible, the standard HiFi workflow run on the Sequel® II System gives you the highest quality results for both genome assembly and human variant detection projects. However, if you are sample-limited, the Low and Ultra-Low DNA Input Workflows will still provide excellent results.

	Standard HiFi Sequencing	Low DNA Input Sequencing 2-Plex	Low DNA Input Sequencing Single Sample	Ultra-Low DNA Input Sequencing
Minimum DNA Input	>3 µg / 1 Gb genome	300 ng for each genome	400 ng	5 ng
Amplification Based?	No	No	No	Yes
Genome Size Limit	N/A	600 Mb for each genome	1 Gb	500 Mb
Supported Applications	*de novo* Assembly Human Variant Detection	*de novo* Assembly	*de novo* Assembly	*de novo* Assembly Human Variant Detection

Table 1. Details of standard, low DNA input, and ultra-low DNA input HiFi sequencing workflows on the Sequel II System.

Whole Genome Sequencing for *de novo* Assembly

For *de novo* genome assembly projects, consider the size of the genome to be sequenced as well as the amount of DNA available when choosing a workflow. The minimum DNA amount for the Low DNA Input Workflow is 300 ng for a 2-plex project where each genome can be up to ~600 Mb in size. If you have multiple genomes of interest that fit within these DNA and genome size requirements, this is an efficient and cost-effective option. If the genome is slightly larger, up to 1 Gb in size, and you are able to extract ≥400 ng of DNA from the organism, the single-sample Low DNA Input Workflow is the appropriate workflow. Both single-sample and 2-plex workflows can be found in the Low DNA Input Protocol.

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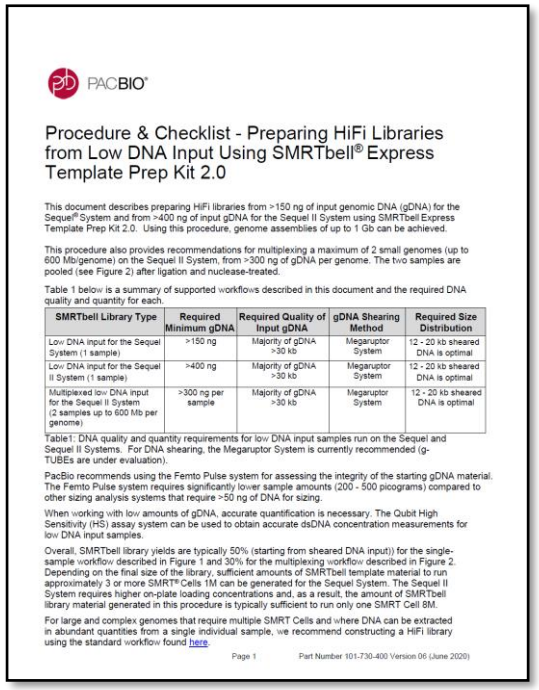
Application Note: Considerations for Using the Low and Ultra-low DNA Input Workflows for Whole Genome Sequencing (101-995-900)

Summary overview of experimental design considerations, sample preparation workflow and data analysis recommendations



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Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell® Express Template Prep Kit 2.0

This document describes preparing HiFi libraries from >150 ng of input genomic DNA (gDNA) for the Sequel® System and from >400 ng of input gDNA for the Sequel II System using SMRTbell Express Template Prep Kit 2.0. Using this procedure, genome assemblies of up to 1 Gb can be achieved.

This procedure also provides recommendations for multiplexing a maximum of 2 small genomes (up to 600 Mb/genome) on the Sequel II System, from >300 ng of gDNA per genome. The two samples are pooled (see Figure 2) after ligation and nuclease-treated.

Table 1 below is a summary of supported workflows described in this document and the required DNA quality and quantity for each.

SMRTbell Library Type	Required Minimum gDNA	Required Quality of Input gDNA	gDNA Shearing Method	Required Size Distribution
Low DNA input for the Sequel System (1 sample)	>150 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Low DNA input for the Sequel II System (1 sample)	>400 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Multiplexed low DNA input for the Sequel II System (2 samples up to 600 Mb per genome)	>300 ng per sample	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal

Table1: DNA quality and quantity requirements for low DNA input samples run on the Sequel and Sequel II Systems. For DNA shearing, the Megaruptor System is currently recommended (g-TUBEs are under evaluation).

PacBio recommends using the Femto Pulse system for assessing the integrity of the starting gDNA material. The Femto Pulse system requires significantly lower sample amounts (200 - 500 picograms) compared to other sizing analysis systems that require >50 ng of DNA for sizing.

When working with low amounts of gDNA, accurate quantification is necessary. The Qubit High Sensitivity (HS) assay system can be used to obtain accurate dsDNA concentration measurements for low DNA input samples.

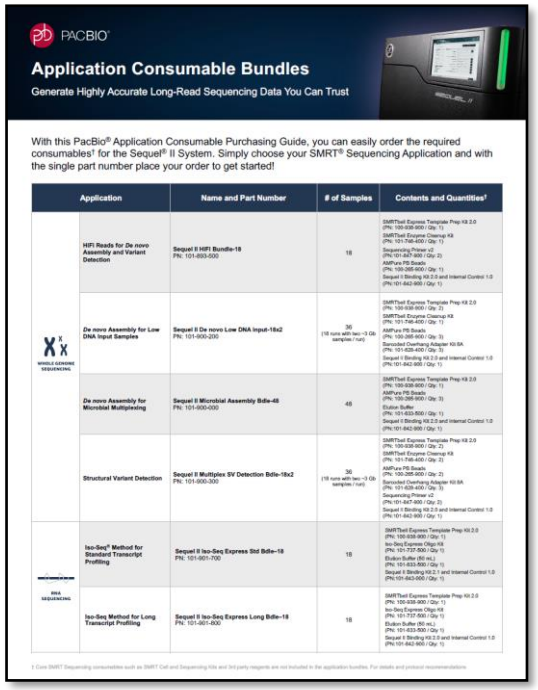
Overall, SMRTbell library yields are typically 50% (starting from sheared DNA input)) for the single-sample workflow described in Figure 1 and 30% for the multiplexing workflow described in Figure 2. Depending on the final size of the library, sufficient amounts of SMRTbell template material to run approximately 3 or more SMRT® Cells 1M can be generated for the Sequel System. The Sequel II System requires higher on-plate loading concentrations and, as a result, the amount of SMRTbell library material generated in this procedure is typically sufficient to run only one SMRT Cell 8M.

For large and complex genomes that require multiple SMRT Cells and where DNA can be extracted in abundant quantities from a single individual sample, we recommend constructing a HiFi library using the standard workflow found here.

Page 1 Part Number 101-730-400 Version 06 (June 2020)

Procedure & Checklist – Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0 (101-730-400)

Technical documentation containing sample library construction and sequencing preparation protocol details



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Application Consumable Bundles

Generate Highly Accurate Long-Read Sequencing Data You Can Trust

With this PacBio® Application Consumable Purchasing Guide, you can easily order the required consumables† for the Sequel® II System. Simply choose your SMRT® Sequencing Application and with the single part number place your order to get started!

Application	Name and Part Number	# of Samples	Contents and Quantities‡
HiFi Reads for *De novo* Assembly and Variant Detection	Sequel II HiFi Bundle-18 PN: 101-893-500	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) Sequencing Primer v2 (PN: 101-847-900 / Qty: 2) AMPure PB Beads (PN: 100-265-000 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN: 101-842-900 / Qty: 1)
De novo Assembly for Low DNA Input Samples	Sequel II De novo Low DNA Input-18x2 PN: 101-900-200	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN: 101-842-900 / Qty: 1)
De novo Assembly for Microbial Multiplexing	Sequel II Microbial Assembly 8dbe-48 PN: 101-930-000	48	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) AMPure PB Beads (PN: 100-265-900 / Qty: 3) Elution Buffer (PN: 101-633-500 / Qty: 1) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN: 101-842-900 / Qty: 1)
Structural Variant Detection	Sequel II Multiplex SV Detection 8dbe-18x2 PN: 101-930-300	36 (18 runs with two ~3 Gb samples / run)	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 2) SMRTbell Enzyme Cleanup Kit (PN: 101-746-400 / Qty: 2) AMPure PB Beads (PN: 100-265-900 / Qty: 2) Barcoded Overhang Adapter Kit 8A (PN: 101-628-400 / Qty: 3) Sequencing Primer v2 (PN: 101-847-900 / Qty: 2) Sequel II Binding Kit 2.0 and Internal Control 1.0 (PN: 101-842-900 / Qty: 1)
Iso-Seq® Method for Standard Transcript Profiling	Sequel II Iso-Seq Express Std 8dbe-18 PN: 101-901-700	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty: 1) Elution Buffer (50 mL) (PN: 101-633-500 / Qty: 1) Sequel II Binding Kit 2.1 and Internal Control 1.0 (PN: 101-843-000 / Qty: 1)
Iso-Seq Method for Long Transcript Profiling	Sequel II Iso-Seq Express Long 8dbe-18 PN: 101-901-800	18	SMRTbell Express Template Prep Kit 2.0 (PN: 100-938-900 / Qty: 1) Iso-Seq Express Oligo Kit (PN: 101-737-500 / Qty: 1) Elution Buffer (50 mL) (PN: 101-633-500 / Qty: 1) Sequel II Binding Kit 2.1 and Internal Control 1.0 (PN: 101-843-000 / Qty: 1)

‡ Core SMRT Sequencing consumables such as SMRT Cell and Sequencing Kits and 3rd party reagents are not included in the application bundles. For details and protocol recommendations

PacBio Application Consumable Bundle Purchasing Guide (PG100-051320)

Purchasing Guide enables users to easily order required consumables needed to prepare a SMRTbell library to run a specific type of application on the Sequel II and IIe Systems*

gDNA QC & Shearing

12 kb – 20 kb Target DNA Shear Size

Library Construction (SMRTbell Express TPK 2.0)

Multiplex Up To 2 low DNA input samples with the Sequel II and IIe Systems using Barcoded Overhang Adapters (BOA)

HiFi Sequencing

Aim for 10- to 15-fold HiFi Read Coverage per Haplotype for Assembly

Data Analysis (SMRT Link)

Demultiplexing Analysis
Genome Assembly Analysis

* Application Consumable Bundles include reagents for library construction, primer annealing and polymerase binding. Core PacBio-branded SMRT Sequencing consumables (SMRT Cells, Sequencing Kits & SMRT Oil), plastics and other 3rd-party reagents are not included in the application bundles

WHEN IS IT APPROPRIATE TO CONSIDER THE LOW DNA OR ULTRA-LOW DNA INPUT WORKFLOWS?

We recommend considering the genome assembly project as a whole, from DNA extraction to bioinformatics, to establish your experimental design.

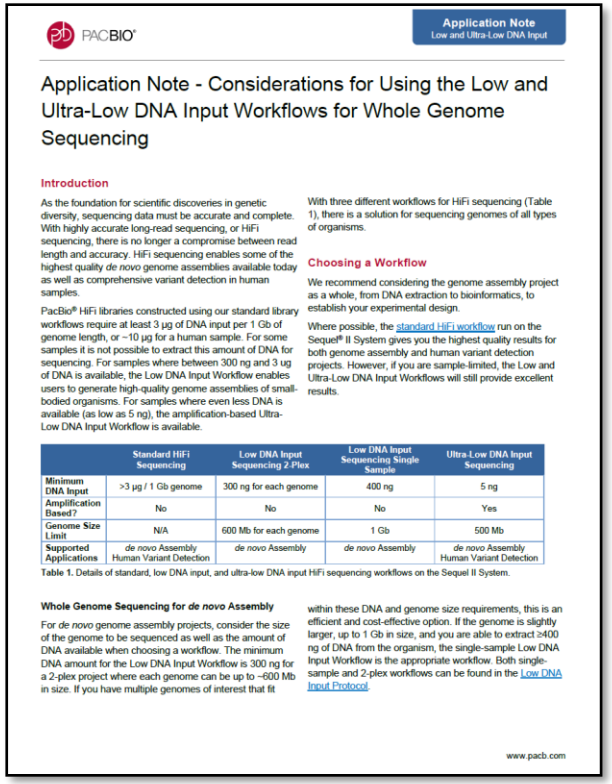
PacBio's standard HiFi library preparation workflow requires at least ~3 µg of DNA input per 1 Gb of genome length, or ~15 µg for a human sample – but for some samples, it is not possible to extract this amount of DNA for sequencing.

For samples where between ~300 ng and ~3 µg of DNA is available, the Low DNA Input Workflow enables users to generate high-quality genome assemblies of small-bodied organisms.

For samples where even less DNA is available (as low as 5 ng), the amplification-based Ultra-Low DNA Input Workflow is available.

- Where possible, the **standard HiFi workflow** run on the Sequel II and IIe Systems gives you the highest quality results for both genome assembly and human variant detection projects
- However, if you are **sample-limited**, the **Low and Ultra-Low DNA Input Workflows** can still provide excellent results.

See **Application Note: Considerations for Using the Low and Ultra-Low DNA Input Workflows for Whole Genome Sequencing (PN 101-995-900)** for further guidance on choosing a specific workflow for sequencing your particular sample type



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Application Note
Low and Ultra-Low DNA Input

Application Note - Considerations for Using the Low and Ultra-Low DNA Input Workflows for Whole Genome Sequencing

Introduction

As the foundation for scientific discoveries in genetic diversity, sequencing data must be accurate and complete. With highly accurate long-read sequencing, or HiFi sequencing, there is no longer a compromise between read length and accuracy. HiFi sequencing enables some of the highest quality *de novo* genome assemblies available today as well as comprehensive variant detection in human samples.

PacBio® HiFi libraries constructed using our standard library workflows require at least 3 µg of DNA input per 1 Gb of genome length, or ~10 µg for a human sample. For some samples it is not possible to extract this amount of DNA for sequencing. For samples where between 300 ng and 3 ug of DNA is available, the Low DNA Input Workflow enables users to generate high-quality genome assemblies of small-bodied organisms. For samples where even less DNA is available (as low as 5 ng), the amplification-based Ultra-Low DNA Input Workflow is available.

With three different workflows for HiFi sequencing (Table 1), there is a solution for sequencing genomes of all types of organisms.

Choosing a Workflow

We recommend considering the genome assembly project as a whole, from DNA extraction to bioinformatics, to establish your experimental design.

Where possible, the standard HiFi workflow run on the Sequel® II System gives you the highest quality results for both genome assembly and human variant detection projects. However, if you are sample-limited, the Low and Ultra-Low DNA Input Workflows will still provide excellent results.

	Standard HiFi Sequencing	Low DNA Input Sequencing 2-Plex	Low DNA Input Sequencing Single Sample	Ultra-Low DNA Input Sequencing
Minimum DNA Input	>3 µg / 1 Gb genome	300 ng for each genome	400 ng	5 ng
Amplification Based?	No	No	No	Yes
Genome Size Limit	N/A	600 Mb for each genome	1 Gb	500 Mb
Supported Applications	*de novo* Assembly Human Variant Detection	*de novo* Assembly	*de novo* Assembly	*de novo* Assembly Human Variant Detection

Table 1. Details of standard, low DNA input, and ultra-low DNA input HiFi sequencing workflows on the Sequel II System.

Whole Genome Sequencing for *de novo* Assembly

For *de novo* genome assembly projects, consider the size of the genome to be sequenced as well as the amount of DNA available when choosing a workflow. The minimum DNA amount for the Low DNA Input Workflow is 300 ng for a 2-plex project where each genome can be up to ~600 Mb in size. If you have multiple genomes of interest that fit within these DNA and genome size requirements, this is an efficient and cost-effective option. If the genome is slightly larger, up to 1 Gb in size, and you are able to extract ≥400 ng of DNA from the organism, the single-sample Low DNA Input Workflow is the appropriate workflow. Both single-sample and 2-plex workflows can be found in the Low DNA Input Protocol.

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WHEN IS IT APPROPRIATE TO CONSIDER THE LOW DNA OR ULTRA-LOW DNA INPUT WORKFLOWS?

DETAILS OF STANDARD, LOW DNA INPUT, AND ULTRA-LOW DNA INPUT HIFI SEQUENCING WORKFLOWS ON THE SEQUEL II SYSTEM.

SAMPLE & PROJECT CONSIDERATIONS	STANDARD HIFI SEQUENCING	LOW DNA INPUT SEQUENCING (2-PLEX)	LOW DNA INPUT SEQUENCING (SINGLE SAMPLE)	ULTRA-LOW DNA INPUT SEQUENCING
Minimum DNA Input	>3 µg / 1 Gb genome	300 ng for each genome	400 ng	5 ng
Amplification Based?	No	No	No	Yes
Genome Size Limit	N/A	600 Mb for each genome	1 Gb	500 Mb
Supported Applications	*De novo* Assembly Human Variant Detection	*De novo* Assembly	*De novo* Assembly	*De novo* Assembly Human Variant Detection

Ultra-Low DNA Input: SUPPORTED APPLICATIONS

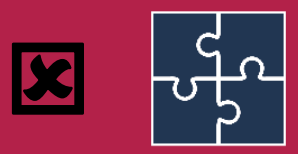
ASSEMBLY

***De novo* assembly of insect/arthropod genomes (Up to 500 Mb)**

VARIANT DETECTION

Variant detection (SNPs, Indels, SVs) in human genomes (3 Gb)

Ultra-Low DNA Input: UNSUPPORTED APPLICATIONS



ASSEMBLY

***De novo* assembly for microbes, plants, vertebrates, or other non-DNA limited sample types**

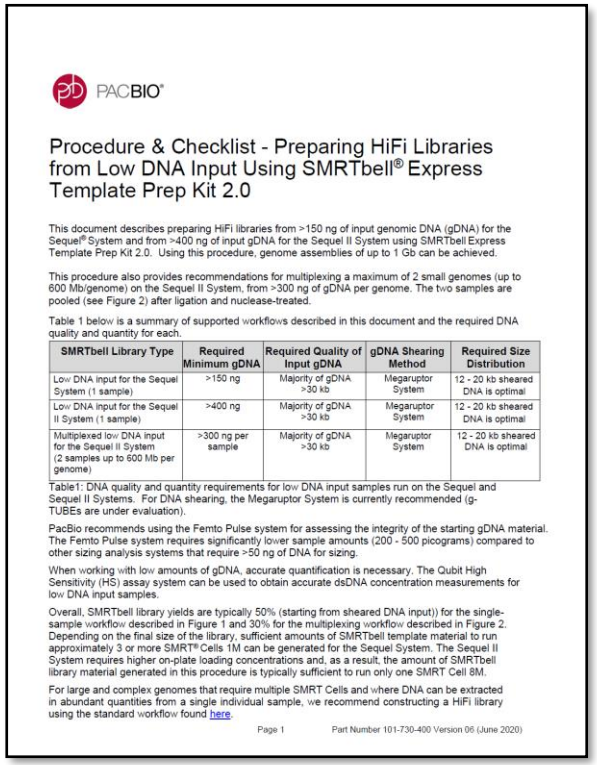
COMPLEX POPULATIONS

Metagenomics sequencing

Low DNA Input Workflow Overview

LOW DNA INPUT SAMPLE PREPARATION PROCEDURE DESCRIPTION

- Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0 (PN 101-730-400) protocol document describes how to prepare SMRTbell libraries with low DNA input amounts for sequencing on the Sequel, Sequel II and Sequel IIe Systems for WGS *de novo* assembly applications using HiFi reads
- The procedure describes preparing HiFi libraries from **>150 ng of input genomic DNA (gDNA) for the Sequel System and from >400 ng of input gDNA for the Sequel II and IIe Systems** using SMRTbell Express TPK 2.0
  - Using this non-multiplexed library preparation procedure, genome assemblies of up to **1 Gb** can be achieved with the Sequel II and IIe Systems
- This procedure also provides recommendations for **multiplexing a maximum of 2 small genomes on the Sequel II or IIe System starting with >300 ng** of gDNA per genome
  - Using this multiplexed library preparation procedure, genome assemblies of up to **600 Mb/genome** can be achieved



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Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell® Express Template Prep Kit 2.0

This document describes preparing HiFi libraries from >150 ng of input genomic DNA (gDNA) for the Sequel® System and from >400 ng of input gDNA for the Sequel II System using SMRTbell Express Template Prep Kit 2.0. Using this procedure, genome assemblies of up to 1 Gb can be achieved.

This procedure also provides recommendations for multiplexing a maximum of 2 small genomes (up to 600 Mb/genome) on the Sequel II System, from >300 ng of gDNA per genome. The two samples are pooled (see Figure 2) after ligation and nuclease-treated.

Table 1 below is a summary of supported workflows described in this document and the required DNA quality and quantity for each.

SMRTbell Library Type	Required Minimum gDNA	Required Quality of Input gDNA	gDNA Shearing Method	Required Size Distribution
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Low DNA input for the Sequel II System (1 sample)	>400 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Multiplexed low DNA input for the Sequel II System (2 samples up to 600 Mb per genome)	>300 ng per sample	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal

Table1: DNA quality and quantity requirements for low DNA input samples run on the Sequel and Sequel II Systems. For DNA shearing, the Megaruptor System is currently recommended (g-TUBEs are under evaluation).

PacBio recommends using the Femto Pulse system for assessing the integrity of the starting gDNA material. The Femto Pulse system requires significantly lower sample amounts (200 - 500 picograms) compared to other sizing analysis systems that require >50 ng of DNA for sizing.

When working with low amounts of gDNA, accurate quantification is necessary. The Qubit High Sensitivity (HS) assay system can be used to obtain accurate dsDNA concentration measurements for low DNA input samples.

Overall, SMRTbell library yields are typically 50% (starting from sheared DNA input)) for the single-sample workflow described in Figure 1 and 30% for the multiplexing workflow described in Figure 2. Depending on the final size of the library, sufficient amounts of SMRTbell template material to run approximately 3 or more SMRT® Cells 1M can be generated for the Sequel System. The Sequel II System requires higher on-plate loading concentrations and, as a result, the amount of SMRTbell library material generated in this procedure is typically sufficient to run only one SMRT Cell 8M.

For large and complex genomes that require multiple SMRT Cells and where DNA can be extracted in abundant quantities from a single individual sample, we recommend constructing a HiFi library using the standard workflow found here.

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Noctua fimbriata
Moth

Anopheles coluzzii
Mosquito

Drosophila erecta
West African Fruit Fly

Oryza sativa subsp. Indica
Rice Plant

Schistosoma mansoni
Parasitic Worm

Vanessa atalanta
Red Admiral Butterfly

APPLICATIONS

WHOLE GENOME SEQUENCING

WORKFLOW SUMMARY OVERVIEW: SINGLE-SAMPLE (NON-MULTIPLEXED) LOW DNA INPUT LIBRARY PREPARATION (SEQUEL, SEQUEL II AND SEQUEL IIe SYSTEMS)

1. Genomic DNA QC and Shearing

- Recommended starting input gDNA quality: >30 kb
- Minimum required input DNA mass per sample for singleplex library construction
  - Sequel System: ≥150 ng
  - Sequel II and IIe Systems: ≥400 ng
- Target DNA shear size: 12 – 20 kb

2. SMRTbell Express TPK 2.0 Library Construction (~4 hours)

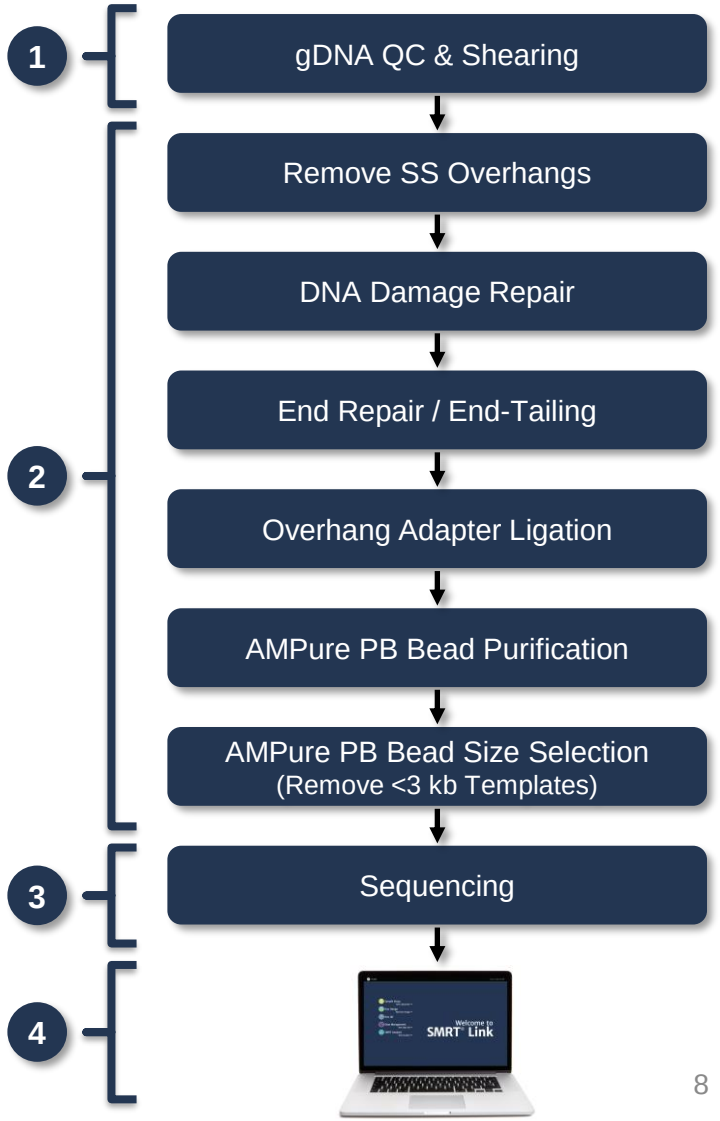
- Follow instructions in ***Procedure & Checklist – Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0*** (PN 101-730-400) for preparation of non-multiplexed samples

3. Sequencing Preparation

- Generate ≥10 Gb of HiFi base data per Sequel II System SMRT Cell 8M* depending on low DNA input sample DNA quality and library insert size

4. Data Analysis

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- For HiFi *de novo* assembly, can use SMRT Link Genome Assembly analysis application (powered by IPA) or other third-party software (e.g., HiCanu, hifiasm)



** Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

WORKFLOW SUMMARY OVERVIEW: MULTIPLEXED LOW DNA INPUT LIBRARY PREPARATION (SEQUEL II AND IIe SYSTEMS)

1. Genomic DNA QC and Shearing

- Recommended starting input gDNA quality: >30 kb
- Minimum required input DNA mass per sample for multiplexed library construction
  - Sequel II and IIe Systems: ≥300 ng per sample
- Target DNA shear size: 12 – 20 kb

2. SMRTbell Express TPK 2.0 Library Construction (~4 hours)

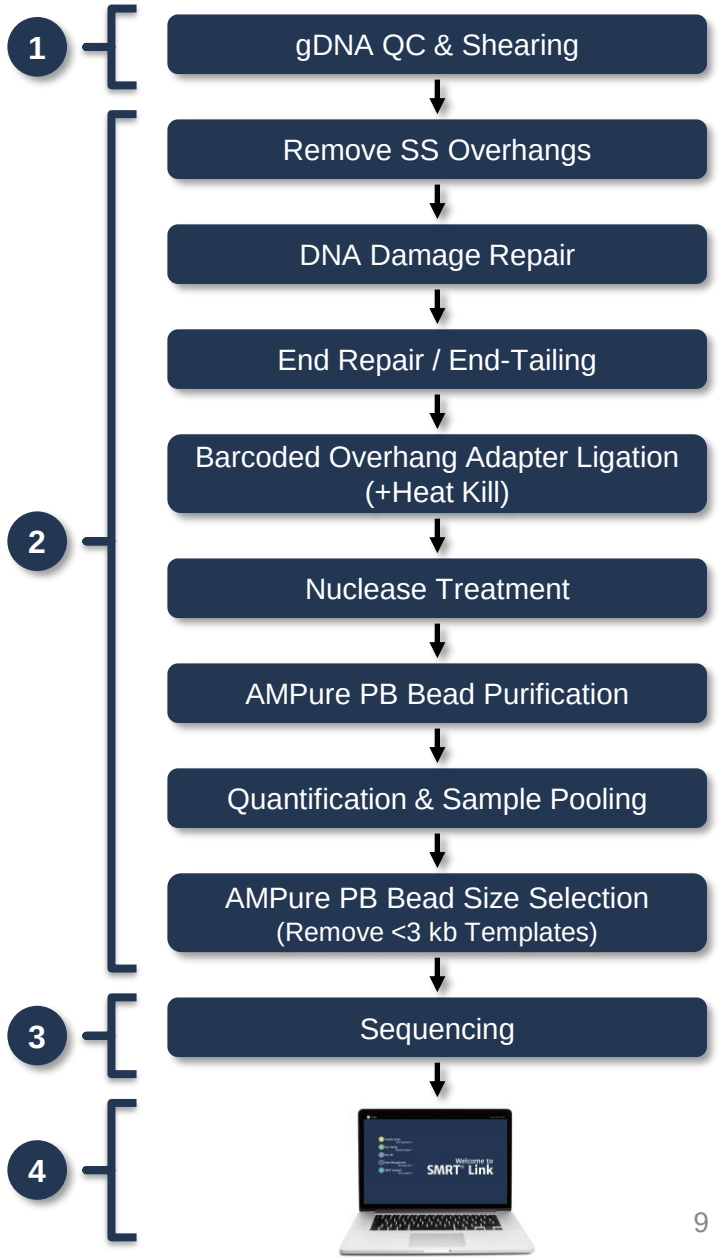
- Follow instructions in ***Procedure & Checklist – Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0*** (PN 101-730-400) for preparation of multiplexed samples
  - Use PacBio Barcoded Overhang Adapter Kit 8A//8B (PN 101-628-400/500) to multiplex up to 2 samples per SMRT Cell 8M

3. Sequencing Preparation

- Generate ≥10 Gb of HiFi base data per Sequel II System SMRT Cell 8M* depending on low DNA input sample DNA quality and library insert size

4. Data Analysis

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (HiFi reads) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- For HiFi *de novo* assembly, can use SMRT Link Genome Assembly analysis application (powered by IPA) or other third-party software



** Read lengths, number of reads, data per SMRT Cell, and other sequencing performance results vary based on sample quality/type and insert size, among other factors.*

Low DNA Input Library Sample QC Requirements

DNA QUALITY AND QUANTITY REQUIREMENTS FOR LOW DNA INPUT SMRTBELL LIBRARY PREPARATION

- Table below summarizes the supported low DNA input workflows and the required DNA quality and quantity for each.

DNA QUALITY AND QUANTITY REQUIREMENTS FOR LOW DNA INPUT SAMPLES RUN ON THE SEQUEL, SEQUEL II AND SEQUEL IIe SYSTEMS. FOR DNA SHEARING, THE MEGARUPTOR SYSTEM IS CURRENTLY RECOMMENDED (COVARIS g-TUBES ARE UNDER EVALUATION).

LOW DNA INPUT LIBRARY TYPE	RECOMMENDED INPUT gDNA AMOUNT	REQUIRED INPUT gDNA QUALITY	gDNA SHEARING METHOD	REQUIRED SHEARED gDNA SIZE DISTRIBUTION
Non-multiplexed low DNA input for the Sequel System 1 sample per SMRT Cell 1M Up to 300 Mb per genome	>150 ng	Majority of gDNA >30 kb	Megaruptor System	12-20 kb sheared gDNA mean fragment size is optimal
Non-multiplexed low DNA input for the Sequel II or IIe Systems 1 sample per SMRT Cell 8M Up to 1 Gb per genome	>400 ng	Majority of gDNA >30 kb	Megaruptor System	12-20 kb sheared gDNA mean fragment size is optimal
Multiplexed low DNA input for the Sequel II or IIe Systems 2 samples per SMRT Cell 8M Up to 600 Mb per genome)	>300 ng per sample	Majority of gDNA >30 kb	Megaruptor System	12-20 kb sheared gDNA mean fragment size is optimal

RECOMMENDED TOOLS FOR GENOMIC DNA QUANTIFICATION AND QUALIFICATION

When working with small amounts of input DNA, accurate sizing and quantification are critical for enabling the generation of sufficient coverage of reads to produce a high-quality genome assembly.

DNA Quantification

- For quantification of gDNA to be used with the low DNA input library preparation workflow, we recommend using the **Qubit fluorometer** and **Qubit High Sensitivity (HS) DNA assay reagents** (Thermo Fisher Scientific)
  - Qubit dsDNA HS assay quantitation range: 0.2 – 100 ng
  - Measure the gDNA sample concentration as recommended by the manufacturer.

Qubit™ dsDNA HS Assay Kit

Qubit 4 Fluorometer

https://www.thermofisher.com/order/catalog/product/Q33230#/Q33230

RECOMMENDED TOOLS FOR GENOMIC DNA QUANTIFICATION AND QUALIFICATION (CONT.)

DNA Sizing

- Three commercially available systems that may be used to evaluate gDNA size distribution are listed below with links to recommended procedures.
  - **Note:** The CHEF Mapper and Pippin Pulse systems **may not be appropriate** options in some cases since both tools require at least 50 ng of DNA sample for analysis

We highly recommend the use of the **Femto Pulse System (Agilent)** for low DNA input applications because of its ability to evaluate size distributions using only ~200 – 500 picograms of DNA

Femto Pulse System

GENOMIC DNA SIZE EVALUATION METHODS AND PROCEDURES.

DNA SIZING METHOD	COMMENTS	PROCEDURE
Femto Pulse System (Agilent)	▪ ***Highly recommended*** ▪ Requires 200 – 500 pg	Agilent Femto Pulse Website
CHEF Mapper XA PFGE System (Bio-Rad)	▪ Requires >50 ng	Procedure & Checklist - Using the BIO-RAD® CHEF Mapper XA Pulsed Field Electrophoresis System
Pippin Pulse System (Sage Science)	▪ Requires >50 ng	Procedure & Checklist - Using the Sage Science Pippin Pulse Electrophoresis Power Supply System

EVALUATION OF GENOMIC DNA FOR LOW DNA INPUT LIBRARY CONSTRUCTION

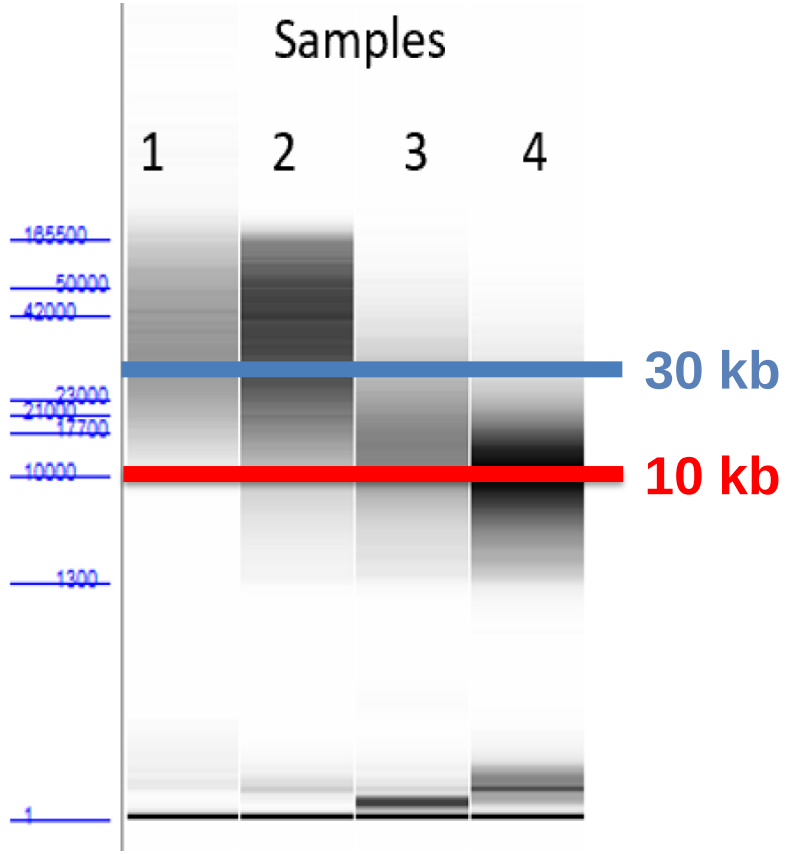
The size distribution of the starting input gDNA sample is critical to successful HiFi SMRTbell library construction and sequencing on the Sequel, Sequel II and Sequel IIe Systems. Always evaluate the quality of the gDNA samples before proceeding with library construction.

- PacBio recommends working with samples where the majority of the input gDNA is **>30 kb**
- SMRTbell libraries constructed from low-quality, fragmented gDNA samples tend to generate shorter read lengths compared to libraries constructed from high-quality, high-molecular weight gDNA samples
  - This may be caused by sequencing termination events occurring at damaged sites introduced during DNA extraction that were not repairable by DNA Damage repair enzymes
  - Additionally, fragmented DNA usually contains excess levels of short fragments (<10 kb) that cannot be removed even with the most aggressive AMPure PB bead size-selection procedure
  - Such types of low-quality DNA samples typically lead to the generation of short subread lengths and – consequently – a more fragmented *de novo* genome assembly

EVALUATION OF GENOMIC DNA FOR LOW DNA INPUT LIBRARY CONSTRUCTION (CONT.)

Example Femto Pulse sizing QC analysis of individual mosquito gDNA samples of varying quality.

- **Samples 1** and **2** show size distributions with the majority of the fragments **>30 kb**.
  - Both samples are appropriate for shearing and constructing HiFi SMRTbell libraries for *de novo* assembly.
- If the gDNA is severely fragmented such that a significant proportion of the fragments are ≤10 kb (e.g., **Samples 3** and **4**), we recommend re-extraction of the gDNA to obtain a higher-quality sample for HiFi SMRTbell library construction.
- If the sample is moderately fragmented with a size distribution of ~10 – 20 kb with relatively few <10 kb fragments, a HiFi SMRTbell library may optionally be constructed directly without shearing (with the caveats noted on the previous slide).

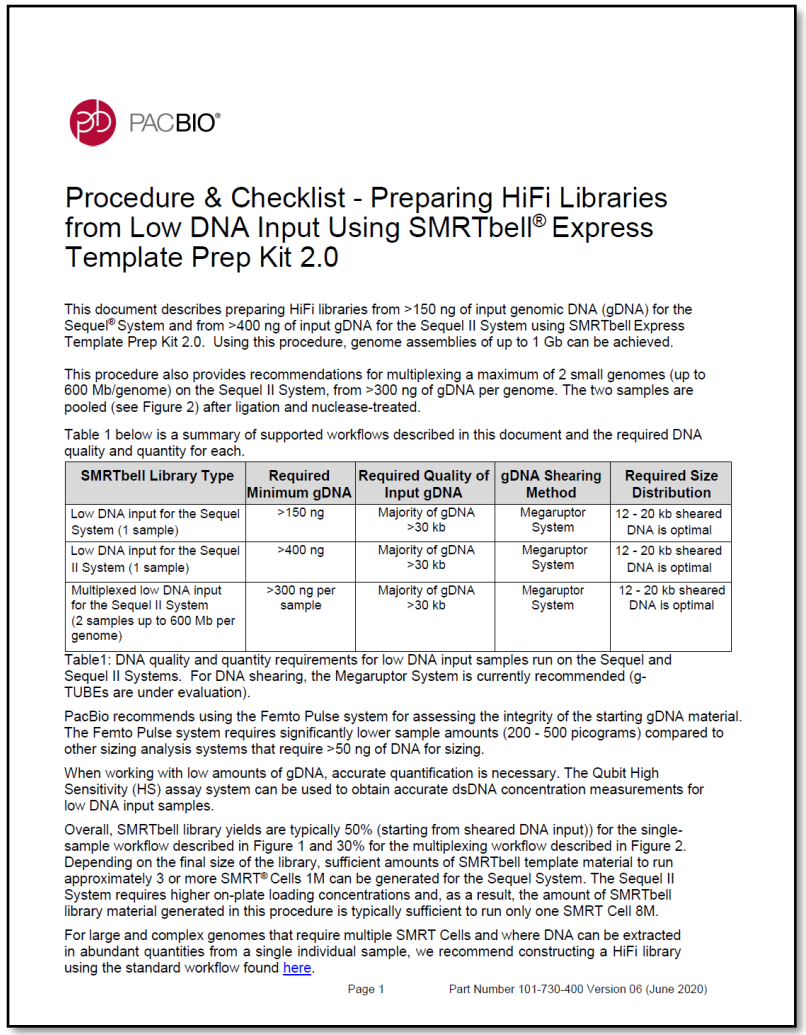


Example Femto Pulse sizing QC analysis of individual mosquito gDNA samples of varying quality. gDNA samples with a majority of fragments >30 kb are suitable for HiFi library construction using this procedure.

Low DNA Input Library Sample Preparation Workflow Details

PROCEDURE & CHECKLIST - PREPARING HIFI LIBRARIES FROM LOW DNA INPUT USING SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0

- This document (PN 101-730-400) describes preparing HiFi libraries from >150 ng of input genomic DNA (gDNA) for the Sequel System and from >400 ng of input gDNA for the Sequel II and IIe Systems using SMRTbell Express TPK 2.0.
  - Using this procedure, genome assemblies of up to 1 Gb can be achieved with the Sequel II and IIe Systems. (Assemblies of up to 300 Mb can be achieved with the Sequel System.)
- This procedure also provides recommendations for multiplexing a maximum of 2 small genomes (up to 600 Mb/genome) on the Sequel II and IIe Systems, from >300 ng of gDNA per genome.
- Protocol document contains:
  1. General laboratory best practices recommendations
  2. Guidelines for QC evaluation of gDNA samples for low-Input library construction
  3. Enzymatic steps for preparation of non-multiplexed and multiplexed low DNA input SMRTbell libraries
  4. Instructions for size-selection of low DNA input libraries using the AMPure PB bead size selection method
  5. Sample setup guidance for preparing low DNA input libraries for sequencing on the Sequel, Sequel II and Sequel IIe Systems



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Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell® Express Template Prep Kit 2.0

This document describes preparing HiFi libraries from >150 ng of input genomic DNA (gDNA) for the Sequel® System and from >400 ng of input gDNA for the Sequel II System using SMRTbell Express Template Prep Kit 2.0. Using this procedure, genome assemblies of up to 1 Gb can be achieved.

This procedure also provides recommendations for multiplexing a maximum of 2 small genomes (up to 600 Mb/genome) on the Sequel II System, from >300 ng of gDNA per genome. The two samples are pooled (see Figure 2) after ligation and nuclease-treated.

Table 1 below is a summary of supported workflows described in this document and the required DNA quality and quantity for each.

SMRTbell Library Type	Required Minimum gDNA	Required Quality of Input gDNA	gDNA Shearing Method	Required Size Distribution
Low DNA input for the Sequel System (1 sample)	>150 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Low DNA input for the Sequel II System (1 sample)	>400 ng	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal
Multiplexed low DNA input for the Sequel II System (2 samples up to 600 Mb per genome)	>300 ng per sample	Majority of gDNA >30 kb	Megaruptor System	12 - 20 kb sheared DNA is optimal

Table1: DNA quality and quantity requirements for low DNA input samples run on the Sequel and Sequel II Systems. For DNA shearing, the Megaruptor System is currently recommended (g-TUBEs are under evaluation).

PacBio recommends using the Femto Pulse system for assessing the integrity of the starting gDNA material. The Femto Pulse system requires significantly lower sample amounts (200 - 500 picograms) compared to other sizing analysis systems that require >50 ng of DNA for sizing.

When working with low amounts of gDNA, accurate quantification is necessary. The Qubit High Sensitivity (HS) assay system can be used to obtain accurate dsDNA concentration measurements for low DNA input samples.

Overall, SMRTbell library yields are typically 50% (starting from sheared DNA input)) for the single-sample workflow described in Figure 1 and 30% for the multiplexing workflow described in Figure 2. Depending on the final size of the library, sufficient amounts of SMRTbell template material to run approximately 3 or more SMRT® Cells 1M can be generated for the Sequel System. The Sequel II System requires higher on-plate loading concentrations and, as a result, the amount of SMRTbell library material generated in this procedure is typically sufficient to run only one SMRT Cell 8M.

For large and complex genomes that require multiple SMRT Cells and where DNA can be extracted in abundant quantities from a single individual sample, we recommend constructing a HiFi library using the standard workflow found here.

Page 1 Part Number 101-730-400 Version 06 (June 2020)

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LIST OF REQUIRED MATERIALS AND EQUIPMENT

ITEM	VENDOR	PART NUMBER
DNA Sizing QC (One of the following)		
Femto Pulse System (**Highly Recommended**)	Agilent	M5330AA
Pippin Pulse Electrophoresis Power Supply	Sage Science	PP10200
Pulsed Field Gel Electrophoresis System: CHEF Mapper XA	Bio-Rad	170-3670
DNA Quantitation		
Qubit Fluorometer	ThermoFisher Scientific	Q33226
Qubit 1X dsDNA HS Assay Kit	ThermoFisher Scientific	Q33230
DNA Shearing		B06010003
Megaruptor 3 System	Diagenode	10145
Megaruptor 3 Shearing Kit	Diagenode	B06010001
SMRTbell Library Preparation		
SMRTbell Express Template Prep Kit 2.0	PacBio	100-938-900
Barcoded Overhang Adapter Kit 8A (8 adapters) or Barcoded Overhang Adapter Kit 8B (8 adapters)	PacBio PacBio	101-628-400 101-628-500
AMPure PB Beads	PacBio	100-265-900
SMRTbell Enzyme Cleanup Kit	PacBio	101-746-400
Elution Buffer	PacBio	101-633-500
DNA Lo Bind microfuge tubes	PacBio	022431021
Wide Orifice Tips (Tips LTS W-O 200UL Fltr RT-L200WFLR)	Rainin	17014294
Tube Rotator	VWR	10136-084

SMRTBELL EXPRESS TEMPLATE PREP KIT 2.0 AND SMRTBELL ENZYME CLEANUP KIT REAGENT HANDLING RECOMMENDATIONS

- Several reagents in the kit are sensitive to temperature and vortexing
- PacBio highly recommends:
  - Never leaving reagents at room temperature
  - Working on ice at all times when preparing master mixes
  - Finger tapping followed by a quick-spin prior to use

SMRTbell Express TPK 2.0

LIST OF TEMPERATURE-SENSITIVE REAGENTS INCLUDED IN SMRTBELL EXPRESS TPK 2.0 AND SMRTBELL ENZYME CLEANUP KIT.

PACBIO KIT	REAGENT	WHERE USED
SMRTbell Express Template Prep kit 2.0 (PN 100-938-900)	DNA Prep Additive	Remove Single-Strand Overhangs
	DNA Prep Enzyme	Remove Single-Strand Overhangs
	DNA Damage Repair Mix v2	DNA Damage Repair
	End Prep Mix	End-Repair/A-tailing
	Overhang Adapter v3*	Ligation
	Ligation Mix	Ligation
	Ligation Additive	Ligation
	Ligation Enhancer	Ligation
SMRTbell Enzyme Cleanup Kit (PN 101-746-400)	Enzyme A	Nuclease Treatment
	Enzyme B	Nuclease Treatment
	Enzyme C	Nuclease Treatment
	Enzyme D	Nuclease Treatment

* Barcoded Overhang Adapters (not included with SMRTbell Express TPK 2.0) are also temperature-sensitive reagents.

SMRTBELL EXPRESS TPK 2.0 LIBRARY CONSTRUCTION WORKFLOW COMPARISON FOR SUPPORTED LOW DNA INPUT LIBRARY TYPES

LIBRARY CONSTRUCTION STEP	LOW DNA INPUT SINGLEPLEX SAMPLE (SEQUEL SYSTEM)	LOW DNA INPUT SINGLEPLEX SAMPLE (SEQUEL II AND IIe SYSTEM)	LOW DNA INPUT MULTIPLEXED SAMPLE (SEQUEL II AND IIe SYSTEM)
Recommended Starting Input gDNA Amount for Shearing	>150 ng (Single Sample)	>400 ng (Single Sample)	>300 ng per sample (Up to 2 Samples)
gDNA Quality	>30 kb		
DNA Shearing	Megaruptor 3 System		
Minimum DNA Input for Remove Single-Stranded DNA Overhangs	>150 ng (Sheared gDNA)	>300 ng (Sheared gDNA)	>200 ng per sample (Sheared gDNA)
Remove Single-Stranded DNA Overhangs	37°C / 15 min		
DNA Damage Repair	37°C / 30 min		
End-Repair/A-tailing	20°C / 10 min; 65°C / 30 min		
Adapter	Overhang Adapter v3	Overhang Adapter v3	Barcoded Overhang Adapter
Adapter Ligation	20°C / 60 min (Overnight ligation optional)		
Nuclease Treatment	Not required	Not required	37°C / 60 min
1st AMPure PB Bead Purification	0.45X AMPure PB Beads	0.45X AMPure PB Beads	0.80X AMPure PB Beads
2nd AMPure PB Bead Purification (Removal of <3 kb SMRTbell Templates)	2.2X AMPure PB Beads (40% v/v)		
Library Construction Yield	50%	50%	30%

BEST PRACTICES RECOMMENDATIONS FOR PREPARING LOW DNA INPUT SMRTBELL LIBRARIES

1. Use wide-bore tips for all mixing steps.
2. Throughout the procedure, **do not flick the tubes to mix**. Flicking induces damage to DNA. Instead, use gentle pipetting with wide-bore tips to mix reagents. Note that template preparation reagents should first be dispensed with a standard pipette tip (i.e., P10 or P20) and then a wide-bore tip should be used for pipette mixing.
3. Never vortex tubes containing high-molecular weight genomic DNA samples.
4. Minimize the number of freeze/thaw cycles the gDNA undergoes to reduce DNA damage.
5. Allowing sufficient time for thawing aliquots of DNA, as partially frozen DNA is prone to shearing.
6. Always set your heat blocks or thermocyclers to the appropriate temperature for incubations before proceeding with the procedure.
7. Ensure that the AMPure PB beads are at **room temperature** prior to performing the purification steps.
8. When performing AMPure PB bead purification steps, note that 80% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 80% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days.
9. Always follow best practices for DNA quantitation using a Qubit fluorometer system. Use the Qubit dsDNA High Sensitivity (HS) reagent kit.

DNA SHEARING RECOMMENDATIONS FOR LOW DNA INPUT LIBRARY CONSTRUCTION

- PacBio recommends the **Megaruptor System (Diagenode)** for shearing genomic DNA because of its ability to generate tight shear distributions and good post-shear recovery efficiencies
- The Megaruptor 3 System is recommended for shearing gDNA to a **target size distribution mode ~12 – 20 kb** for low DNA input applications
- To shear gDNA using the Megaruptor 3 system, generally follow the manufacturer's recommendations
- After shearing the gDNA samples, evaluate the size distribution using a Femto Pulse system and then proceed with the Concentrate DNA Using AMPure PB Beads step

Megaruptor 3 System

RECOMMENDED STARTING INPUT GENOMIC DNA AMOUNTS FOR SHEARING.

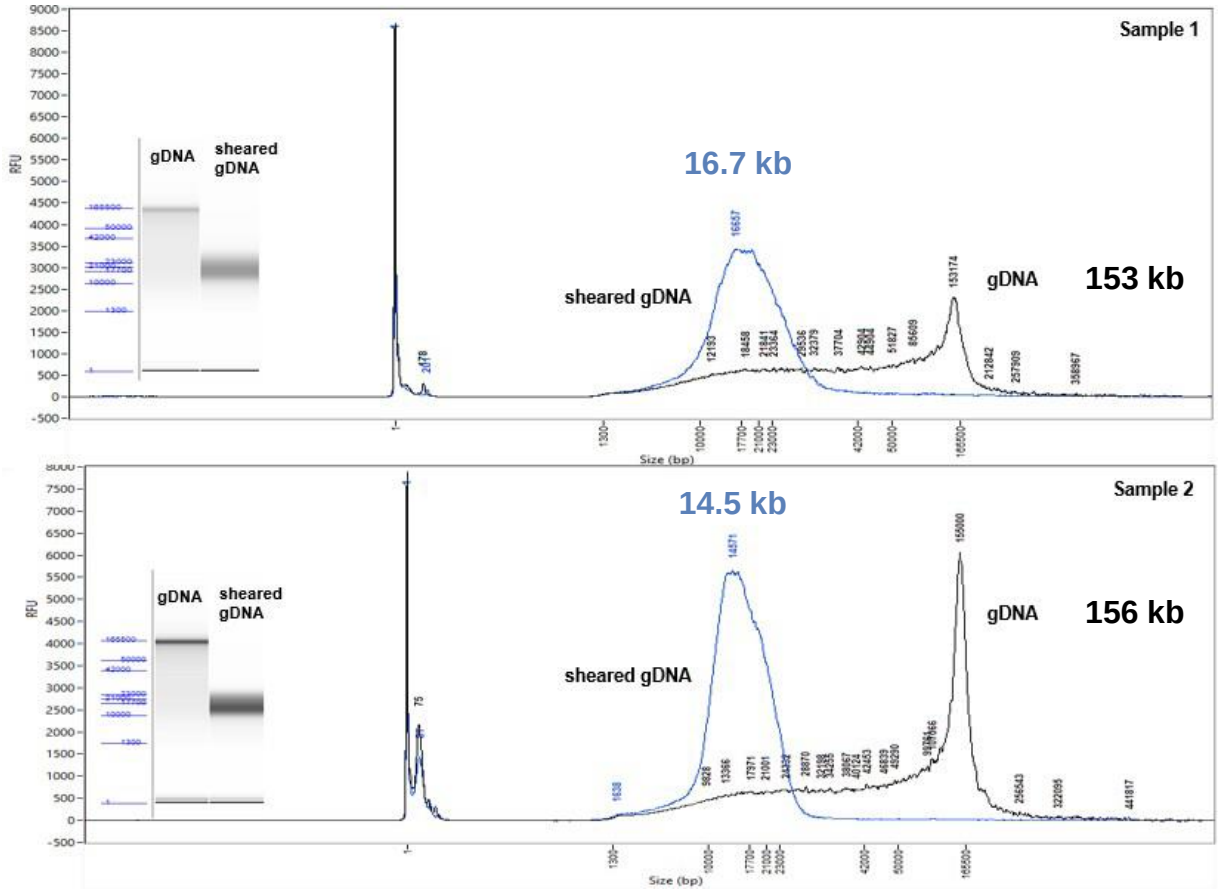
LOW DNA INPUT LIBRARY RECOMMENDATIONS	LOW DNA INPUT SINGLE-SAMPLE (SEQUEL SYSTEM)	LOW DNA INPUT SINGLE-SAMPLE (SEQUEL II AND IIe SYSTEM)	LOW DNA INPUT MULTIPLEXED SAMPLE (SEQUEL II AND IIe SYSTEM)
Recommended Starting Input gDNA Amount for Shearing	**>150 ng** (Single Sample)	**>400 ng** (Single Sample)	**>300 ng per sample** (Up to 2 Samples)

DNA SHEARING RECOMMENDATIONS FOR LOW DNA INPUT LIBRARY CONSTRUCTION (CONT.)

Sheared gDNA QC considerations when multiplexing low DNA input samples on the Sequel II and IIe Systems

For constructing <u>multiplexed</u> low DNA input HiFi SMRTbell libraries for the Sequel II and IIe Systems, we recommend the following:

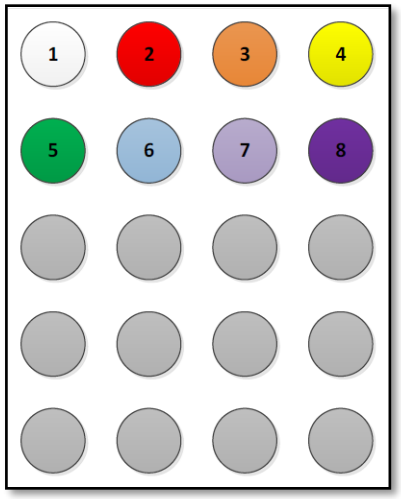
- It is necessary to shear the gDNA so that the fragment size distribution is **12 kb – 20 kb**.
  - This insert size range maximizes the detection of barcodes during the demultiplexing analysis step.
- Samples for a multiplex experiment must have the **same average size and distribution** to avoid biased read representation.
  - It is highly recommended to work with samples that contain mostly high-molecular weight DNA so that they can be sheared to achieve a similar target size and distribution for HiFi SMRTbell library construction.



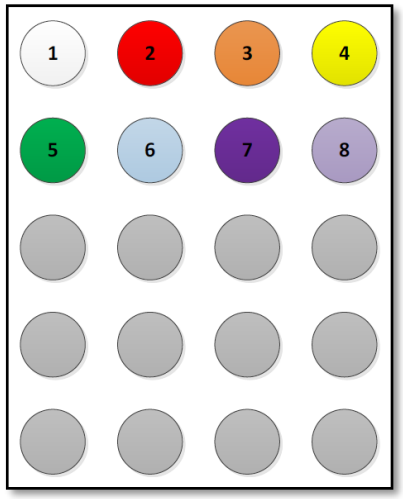
Example Femto Pulse sizing QC analysis of input gDNA and sheared gDNA samples. Samples 1 and 2 are examples of gDNA suitable for library construction using the low DNA input procedure for multiplexing. Both samples were sheared using a Megaruptor 3 system with speed setting 33. Sample 1 sheared DNA mode size is ~16 kb and Sample 2 is ~14 kb.

PACBIO BARCODED OVERHANG ADAPTERS FOR MULTIPLEXED LOW DNA INPUT LIBRARY CONSTRUCTION

- **PacBio Barcoded Overhang Adapter Kit 8A** (PN 101-628-400) **or 8B** (PN 101-628-500) are available for multiplexing low DNA input samples.
  - 8 barcodes in each kit; 6 reactions per BOA tube
  - Compatible with SMRTbell Express TPK 2.0
  - Includes Sequencing Primer v4



Barcoded Overhang Adapter Kit - 8A (PN 101-628-400)	
Tube #	**Description**
1	TUBE, Bar Over Adapt - bc1001
2	TUBE, Bar Over Adapt - bc1002
3	TUBE, Bar Over Adapt - bc1003
4	TUBE, Bar Over Adapt - bc1008
5	TUBE, Bar Over Adapt - bc1009
6	TUBE, Bar Over Adapt - bc1010
7	TUBE, Bar Over Adapt - bc1011
8	TUBE, Bar Over Adapt - bc1012



Barcoded Overhang Adapter Kit - 8B (PN 101-628-500)	
Tube #	**Description**
1	TUBE, Bar Over Adapt - bc1015
2	TUBE, Bar Over Adapt - bc1016
3	TUBE, Bar Over Adapt - bc1017
4	TUBE, Bar Over Adapt - bc1018
5	TUBE, Bar Over Adapt - bc1019
6	TUBE, Bar Over Adapt - bc1020
7	TUBE, Bar Over Adapt - bc1021
8	TUBE, Bar Over Adapt - bc1022

- To download the barcode FASTA sequences for BOA Kit 8A/8B, visit PacBio's Multiplexing Resources webpage
  - FASTA filename: Sequel_16_Barcodes_v3.zip (Link)

SAMPLE POOLING BEST PRACTICES RECOMMENDATIONS FOR MULTIPLEXED LOW DNA INPUT LIBRARY CONSTRUCTION

- **Always quantify libraries before pooling.**
  - Since DNA is in limited quantity, PacBio recommends using the Qubit dsDNA High Sensitivity (HS) Assay Kit for concentration measurements.
- **Equal mass pooling** is recommended for samples that have a similar distribution (+/- 10%)
  - The total mass of the pooled library must be **≥150 ng** (e.g. 75 sample 1 + 75 ng sample 2)
  - If the total mass of the two samples is <150 ng, use the remaining sample to adjust the total mass to 150 ng.
    - This is to ensure that there is enough library material for loading at least one SMRT Cell 8M
    - In such cases, it is possible that the resulting number of reads per sample may be slightly imbalanced
- After pooling, **perform AMPure PB bead size selection to remove fragments <3 kb**

STEP	✓	Pooling	Notes
1		Pool barcoded libraries into a single 1.5 mL DNA Lo-Bind tube.	
2		Mix and spin down the contents of the tube with a quick spin in a microfuge.	
3		Bring the total volume of the pooled sample to 100 µL with Elution Buffer.	
4		Proceed to the **"Size-Selection with AMPure PB Beads to remove <3 kb SMRTbell Templates"** section below.	

AMPURE PB BEAD SIZE SELECTION FOR LOW DNA INPUT LIBRARY CONSTRUCTION

- This final AMPure PB bead purification step removes SMRTbell templates <3 kb
- AMPure PB bead stock solution is first diluted to 40% (vol./vol.) with Elution Buffer and subsequently used for purification
- **The final AMPure PB bead concentration is critical to the success of this procedure.**
  - Therefore, accurate pipetting is of utmost importance to achieve a final 40% (v/v) AMPure PB bead working solution in EB

Reagent	Volume	✓	Notes
Elution Buffer	3.0 mL		
AMPure PB Bead (stock reagent, thoroughly mixed)	2.0 mL		
Total Volume	5.0 mL		

1. Bring the AMPure PB bead stock solution to room temperature.
2. Vortex the stock solution for 30 seconds to mix well.
3. Using a P1000 pipette, transfer 3.0 mL of Elution Buffer into a 15 mL conical tube.
4. Add 2.0 mL of the AMPure PB bead stock solution to the 3.0 mL of Elution Buffer. When pipetting the viscous AMPure PB bead stock solution, pipette slowly to ensure that the volume aspirated is as precise as possible. Large residual AMPure solution adhering to the tip should be removed prior to addition to the 3.0 mL of Elution Buffer.
5. Vortex the diluted AMPure PB bead solution for 30 seconds to mix well before use. This solution may be stored at 4°C for 2 weeks for future use.

LOW DNA INPUT SMRTBELL LIBRARY CONSTRUCTION YIELDS

- Overall, SMRTbell library yields in this low-input workflow are typically **50%** (starting from sheared DNA input into the first enzymatic reaction) for the single-sample workflow and **30%** for the multiplexing workflow
- Depending on the final size of the library, sufficient amounts of SMRTbell template material to run approximately **3 or more SMRT Cells 1M** can be generated for the **Sequel System**
- The **Sequel II and IIe Systems** require higher on-plate loading concentrations and, as a result, the amount of SMRTbell library material generated in this procedure is enough for only **<u>one</u> SMRT Cell 8M**

Low DNA Input Library Sequencing Workflow Details

SAMPLE SETUP RECOMMENDATIONS FOR LOW DNA INPUT LIBRARIES – SEQUEL SYSTEM (CHEMISTRY 3.0 / SMRT LINK V9.0)

- Follow **SMRT Link v9.0 Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel System for low DNA input samples with the additional guidance noted below

Applications	Sequencing Mode	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
Large insert (>15 kb size-selection cutoff)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	2 - 8
Microbial Multiplex (10 kb)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	2 - 8
Low DNA Input (>10 kb, AMPure PB Bead size-selection)	CLR	Express Prep 2.0	Binding Kit 3.0	v4	1	1.2X AMPure PB Beads	4 - 8

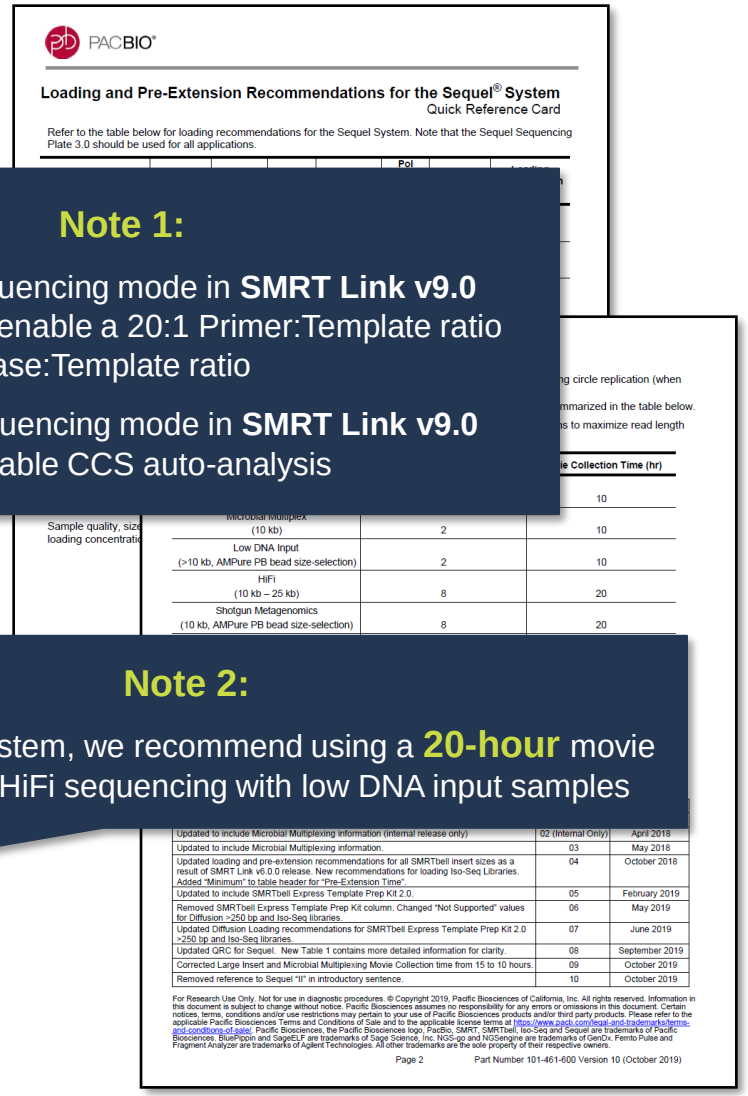
Note 1:

- Select "**CLR**" sequencing mode in **SMRT Link v9.0 Sample Setup** to enable a 20:1 Primer:Template ratio and 30:1 Polymerase:Template ratio
- Select "**CCS**" sequencing mode in **SMRT Link v9.0 Run Design** to enable CCS auto-analysis

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
Large insert (>15 kb size-selection cutoff)	0	10
Microbial Multiplex (10 kb)	2	10
Low DNA Input (>10 kb, AMPure PB bead size-selection)	2	10

Note 2:

- For the Sequel System, we recommend using a **20-hour** movie collection time for HiFi sequencing with low DNA input samples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Loading and Pre-Extension Recommendations for the Sequel® System
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel System. Note that the Sequel Sequencing Plate 3.0 should be used for all applications.

	Pol	
Microbial Multiplex (10 kb)	2	10
Low DNA Input (>10 kb, AMPure PB bead size-selection)	2	10
HiFi (10 kb – 25 kb)	8	20
Shotgun Metagenomics (10 kb, AMPure PB bead size-selection)	8	20

Updated to include Microbial Multiplexing information (internal release only)	02 (Internal Only)	April 2018
Updated to include Microbial Multiplexing information.	03	May 2018
Updated loading and pre-extension recommendations for all SMRTbell insert sizes as a result of SMRT Link v6.0.0 release. New recommendations for loading Iso-Seq Libraries. Added "Minimum" to table header for "Pre-Extension Time".	04	October 2018
Updated to include SMRTbell Express Template Prep Kit 2.0.	05	February 2019
Removed SMRTbell Express Template Prep Kit column. Changed "Not Supported" values for Diffusion >250 bp and Iso-Seq libraries.	06	May 2019
Updated Diffusion Loading recommendations for SMRTbell Express Template Prep Kit 2.0 >250 bp and Iso-Seq libraries.	07	June 2019
Updated QRC for Sequel. New Table 1 contains more detailed information for clarity.	08	September 2019
Corrected Large Insert and Microbial Multiplexing Movie Collection time from 15 to 10 hours.	09	October 2019
Removed reference to Sequel "II" in introductory sentence.	10	October 2019

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SAMPLE SETUP RECOMMENDATIONS FOR LOW DNA INPUT LIBRARIES – SEQUEL II SYSTEM (CHEMISTRY 2.0 / SMRT LINK V9.0)

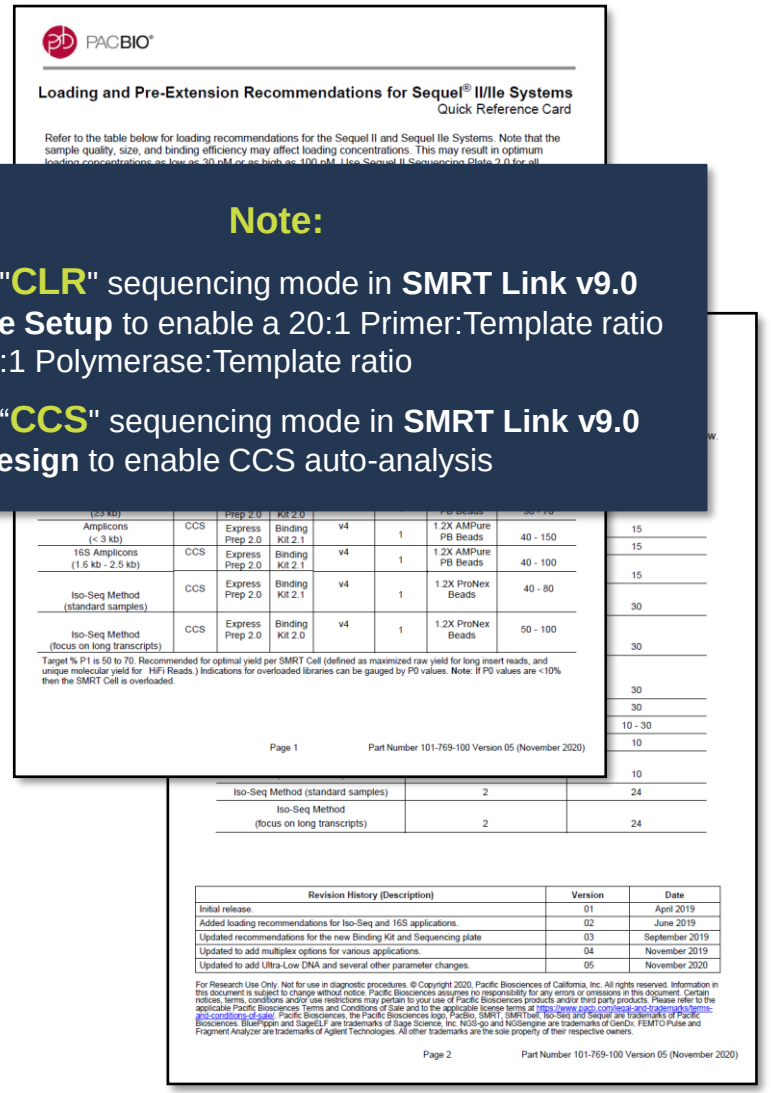
- Follow **SMRT Link v9.0 Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II System for low DNA input samples with the additional guidance noted below

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4		PB Beads	70 – 100
De Novo Assembly – Low DNA Input (15 kb)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 – 70

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly – Continuous Long Reads (>15 kb)	0	15
Structural Variation Detection	2 hrs (≤20 kb), 4 hrs (>20 kb)	15
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	15
De Novo Assembly – Low DNA Input (15 kb)	2	30

Note:

- Select "**CLR**" sequencing mode in **SMRT Link v9.0 Sample Setup** to enable a 20:1 Primer:Template ratio and 30:1 Polymerase:Template ratio
- Select "**CCS**" sequencing mode in **SMRT Link v9.0 Run Design** to enable CCS auto-analysis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Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 30 pM or as high as 100 pM. Use Sequel II Sequencing Plate 2.0 for all

Amplicons (< 3 kb)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 150	15
16S Amplicons (1.6 kb - 2.5 kb)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X AMPure PB Beads	40 - 100	15
Iso-Seq Method (standard samples)	CCS	Express Prep 2.0	Binding Kit 2.1	v4	1	1.2X ProNex Beads	40 - 80	15
Iso-Seq Method (focus on long transcripts)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X ProNex Beads	50 - 100	30

Target % P1 is 50 to 70. Recommended for optimal yield per SMRT Cell (defined as maximized raw yield for long insert reads, and unique molecular yield for HiFi Reads.) Indications for overloaded libraries can be gauged by P0 values. Note: If P0 values are <10% then the SMRT Cell is overloaded.

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Iso-Seq Method (standard samples)	2	24	30
Iso-Seq Method (focus on long transcripts)	2	24	30
			30
			10 - 30
			10
			10

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020

Page 2 Part Number 101-769-100 Version 05 (November 2020)

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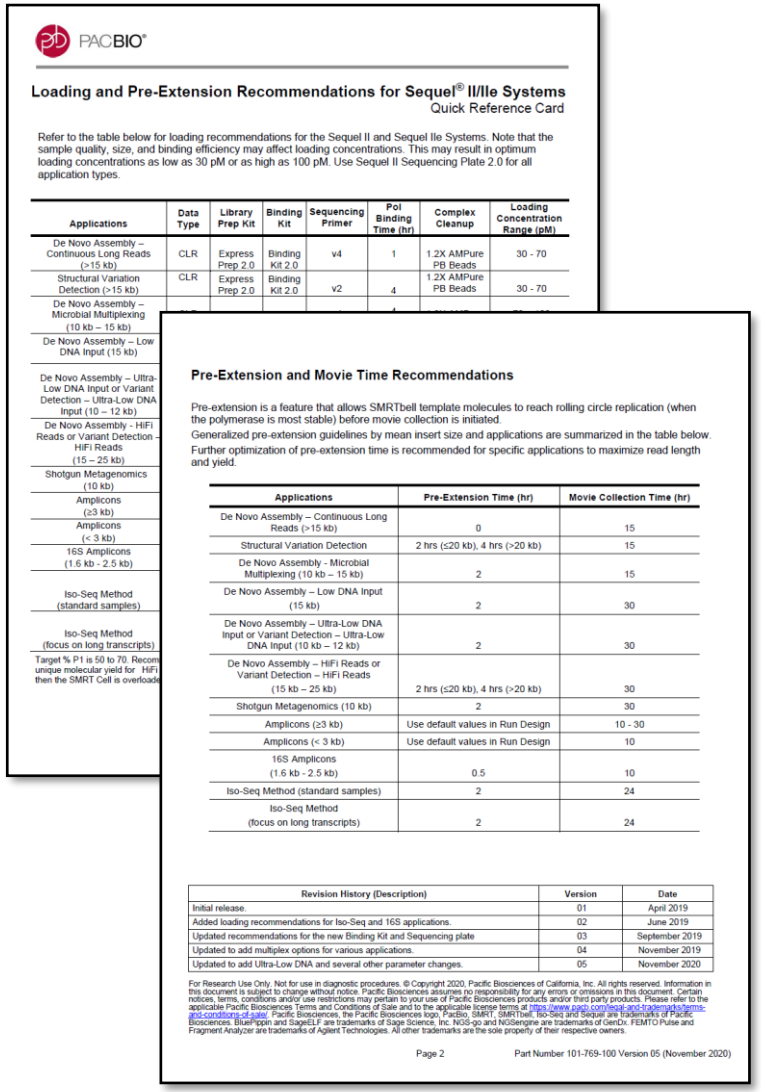
SAMPLE SETUP RECOMMENDATIONS FOR LOW DNA INPUT LIBRARIES – SEQUEL II AND IIe SYSTEMS (CHEMISTRY 2.0 / SMRT LINK V10.0)

- Follow **SMRT Link v10.0 Sample Setup** instructions using the recommendations provided in the Quick Reference Card – Loading and Pre-Extension Time Recommendations for the Sequel II/IIe Systems for preparing Low DNA Input library samples for sequencing

  → For **SMRT Link v10.0** (or higher): Select **'Low DNA Input'** from the **Application** field drop-down menu in the SMRT Link Sample Setup and SMRT Link Run Design user interface

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 - 70
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	4	1.2X AMPure PB Beads	70 – 100
De Novo Assembly – Low DNA Input (15 kb)	CCS	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 – 70

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly – Continuous Long Reads (>15 kb)	0	15
Structural Variation Detection	2 hrs (≤20 kb), 4 hrs (>20 kb)	15
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	15
De Novo Assembly – Low DNA Input (15 kb)	2	30


PACBIO®

Loading and Pre-Extension Recommendations for Sequel® II/IIe Systems
Quick Reference Card

Refer to the table below for loading recommendations for the Sequel II and Sequel IIe Systems. Note that the sample quality, size, and binding efficiency may affect loading concentrations. This may result in optimum loading concentrations as low as 30 pM or as high as 100 pM. Use Sequel II Sequencing Plate 2.0 for all application types.

Applications	Data Type	Library Prep Kit	Binding Kit	Sequencing Primer	Pol Binding Time (hr)	Complex Cleanup	Loading Concentration Range (pM)
De Novo Assembly – Continuous Long Reads (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v4	1	1.2X AMPure PB Beads	30 - 70
Structural Variation Detection (>15 kb)	CLR	Express Prep 2.0	Binding Kit 2.0	v2	4	1.2X AMPure PB Beads	30 - 70
De Novo Assembly – Microbial Multiplexing (10 kb – 15 kb)							
De Novo Assembly – Low DNA Input (15 kb)							
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 – 12 kb)							
De Novo Assembly - HiFi Reads or Variant Detection – HiFi Reads (15 – 25 kb)							
Shotgun Metagenomics (10 kb)							
Amplicons (≥3 kb)							
Amplicons (< 3 kb)							
16S Amplicons (1.6 kb - 2.5 kb)							
Iso-Seq Method (standard samples)							
Iso-Seq Method (focus on long transcripts)							

Target % P1 is 50 to 70. Recom
unique molecular yield for HiFi
then the SMRT Cell is overload

Pre-Extension and Movie Time Recommendations

Pre-extension is a feature that allows SMRTbell template molecules to reach rolling circle replication (when the polymerase is most stable) before movie collection is initiated.
Generalized pre-extension guidelines by mean insert size and applications are summarized in the table below.
Further optimization of pre-extension time is recommended for specific applications to maximize read length and yield.

Applications	Pre-Extension Time (hr)	Movie Collection Time (hr)
De Novo Assembly – Continuous Long Reads (>15 kb)	0	15
Structural Variation Detection	2 hrs (≤20 kb), 4 hrs (>20 kb)	15
De Novo Assembly - Microbial Multiplexing (10 kb – 15 kb)	2	15
De Novo Assembly – Low DNA Input (15 kb)	2	30
De Novo Assembly – Ultra-Low DNA Input or Variant Detection – Ultra-Low DNA Input (10 kb – 12 kb)	2	30
De Novo Assembly – HiFi Reads or Variant Detection – HiFi Reads (15 kb – 25 kb)	2 hrs (≤20 kb), 4 hrs (>20 kb)	30
Shotgun Metagenomics (10 kb)	2	30
Amplicons (≥3 kb)	Use default values in Run Design	10 - 30
Amplicons (< 3 kb)	Use default values in Run Design	10
16S Amplicons (1.6 kb - 2.5 kb)	0.5	10
Iso-Seq Method (standard samples)	2	24
Iso-Seq Method (focus on long transcripts)	2	24

Revision History (Description)	Version	Date
Initial release.	01	April 2019
Added loading recommendations for Iso-Seq and 16S applications.	02	June 2019
Updated recommendations for the new Binding Kit and Sequencing plate	03	September 2019
Updated to add multiplex options for various applications.	04	November 2019
Updated to add Ultra-Low DNA and several other parameter changes.	05	November 2020

Part Number 101-769-100 Version 05 (November 2020)

https://www.pacb.com/support/documentation/

IMPORTING THE BARCODE FASTA FILE INTO SMRT LINK FOR AUTOMATED DEMULTIPLEXING OF POOLED LOW DNA INPUT LIBRARY SAMPLES

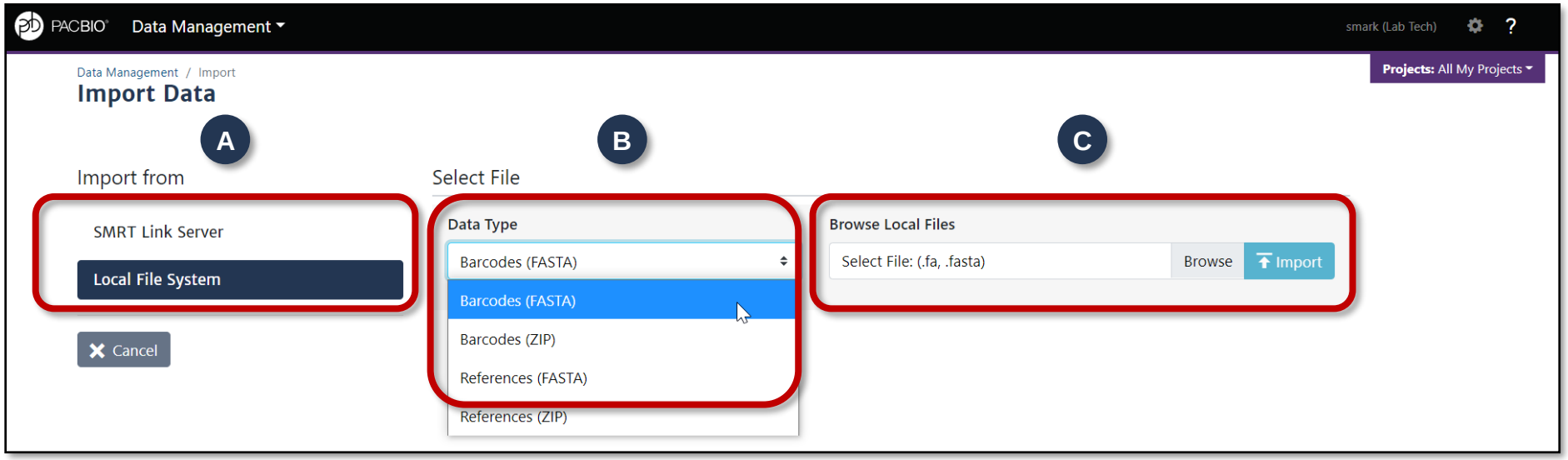
- **Note:** SMRT Link v9.0 (and higher) software installations by default come **pre-bundled** with a FASTA file containing a list of PacBio barcodes recommended for use with multiplexed SMRT sequencing applications
- If your SMRT Link installation does **not** already include an appropriate barcode FASTA file, the following steps describe how to import such a file for use in automated demultiplexing (refer to "Importing Data" section in the SMRT Link User Guide):

1. Download the FASTA file containing the relevant barcode sequences from PacBio's Multiplexing website, for example:
   - Sequel_16_Barcodes_v3.zip (contains a list of 16 PacBio barcodes for use with Barcoded Overhang Adapters)

EXAMPLE FASTA FILE CONTAINING A LIST OF PACBIO 16-BASE PAIR BARCODES

IMPORTING THE BARCODE FASTA FILE INTO SMRT LINK FOR AUTOMATED DEMULTIPLEXING OF POOLED LOW DNA INPUT LIBRARY SAMPLES (CONT.)

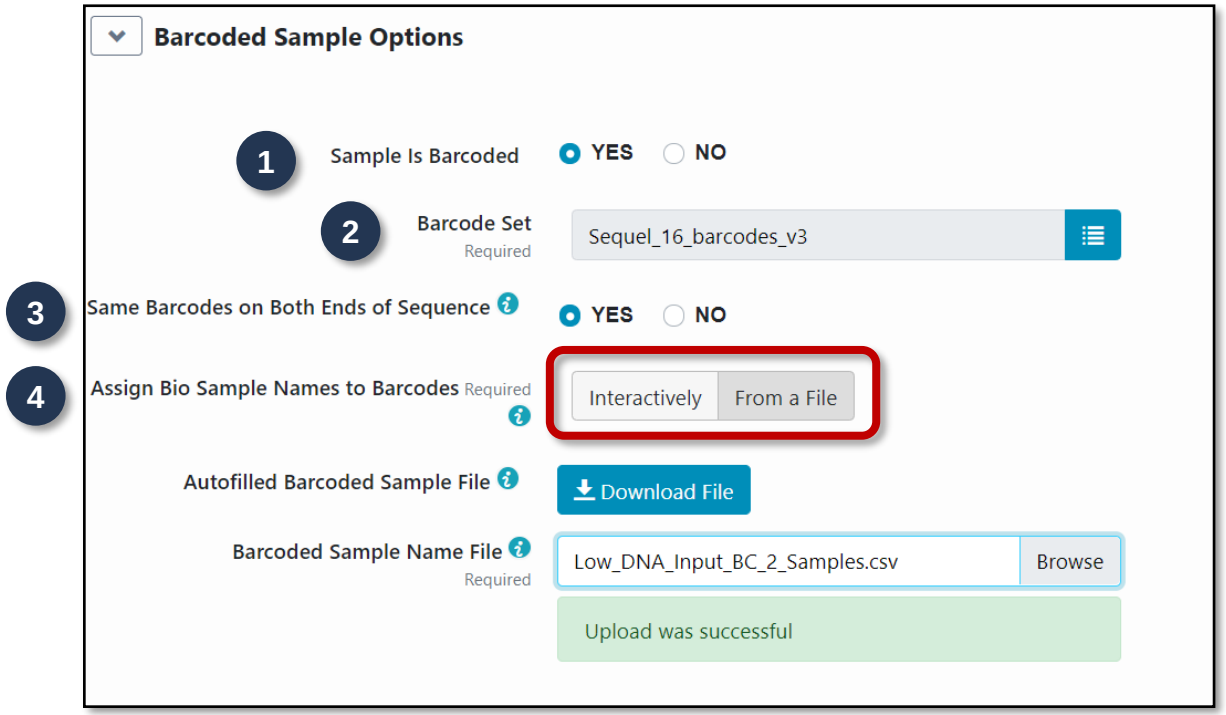
2. Import the desired FASTA file into SMRT Link.
   i. On the SMRT Link Home Page, select **Data Management**.
   ii. Click **Import Data** and follow the steps below:
      A. Specify whether to import data from the **SMRT Link Server**, or from a **Local File System**. (**Note:** Only references and barcodes are available if you select Local File System.)
      B. Select the data type to import: **Barcodes** – FASTA (.fa or .fasta), XML (.barcodeset.xml), or ZIP files containing barcodes.
      C. Navigate to the appropriate file and click **Import**. The selected barcode filed is imported and becomes available for viewing in the SMRT Link Data Management module home screen.



SMRT LINK RUN DESIGN SETUP PROCEDURE FOR AUTOMATED DEMULTIPLEXING OF POLLED LOW DNA INPUT LIBRARY SAMPLES

- Open the Run Design module in SMRT Link and click **New Run Design**.
- Fill in the Sample Information section, then click the small arrow to open **Barcoded Sample Options**.
- Specify the following options:

1. Sample is Barcoded: **Yes**
2. Barcode Set: **<Select Barcode File Name> (e.g., `Sequel_16_barcodes_v3`)**
3. Same Barcodes on Both Ends of Sequence: **Yes**
4. Assign a Biological Sample Name to each barcoded sample using one of two ways: or **From a CSV File** or **Interactively (SMRT Link v10.0 or higher only)**

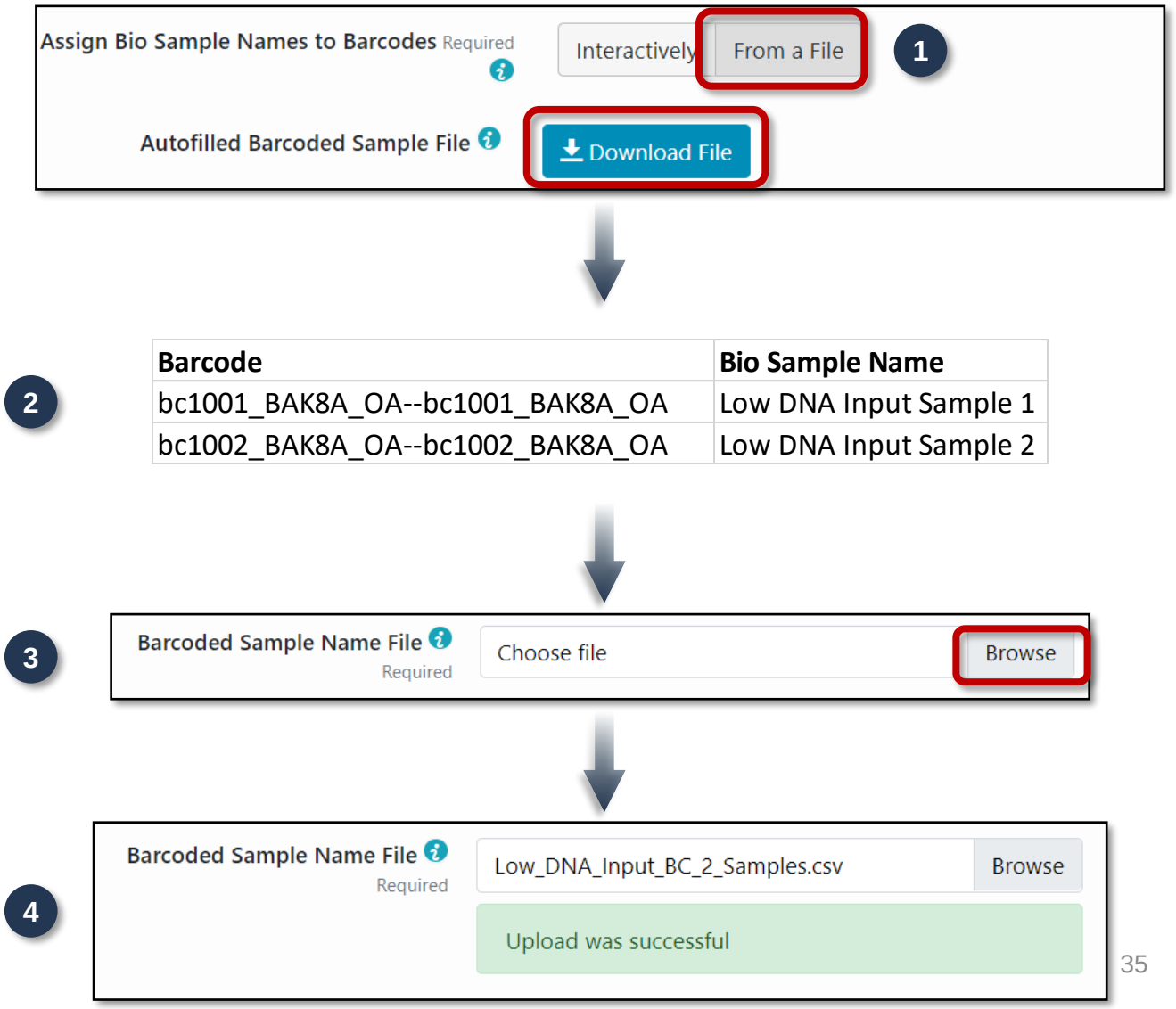


SMRT LINK RUN DESIGN SETUP PROCEDURE FOR AUTOMATED DEMULTIPLEXING OF POOLED LOW DNA INPUT LIBRARY SAMPLES (CONT.)

Barcode Selection and Bio Sample Name Specification Using a CSV File:

1. Click the **From a File** button, then click **Download File**.
2. Edit the file and enter the biological sample names associated with the barcodes in the second column, then save the file.
   - Delete entire rows of barcodes not used
   - Allowed characters: Alphanumeric; space; dot; underscore; hyphen. Other characters will be automatically removed.
3. Browse for the Barcoded Sample File you just edited and click on Open.
4. You see Upload Was Successful appear on the line below, assuming the file is formatted correctly..

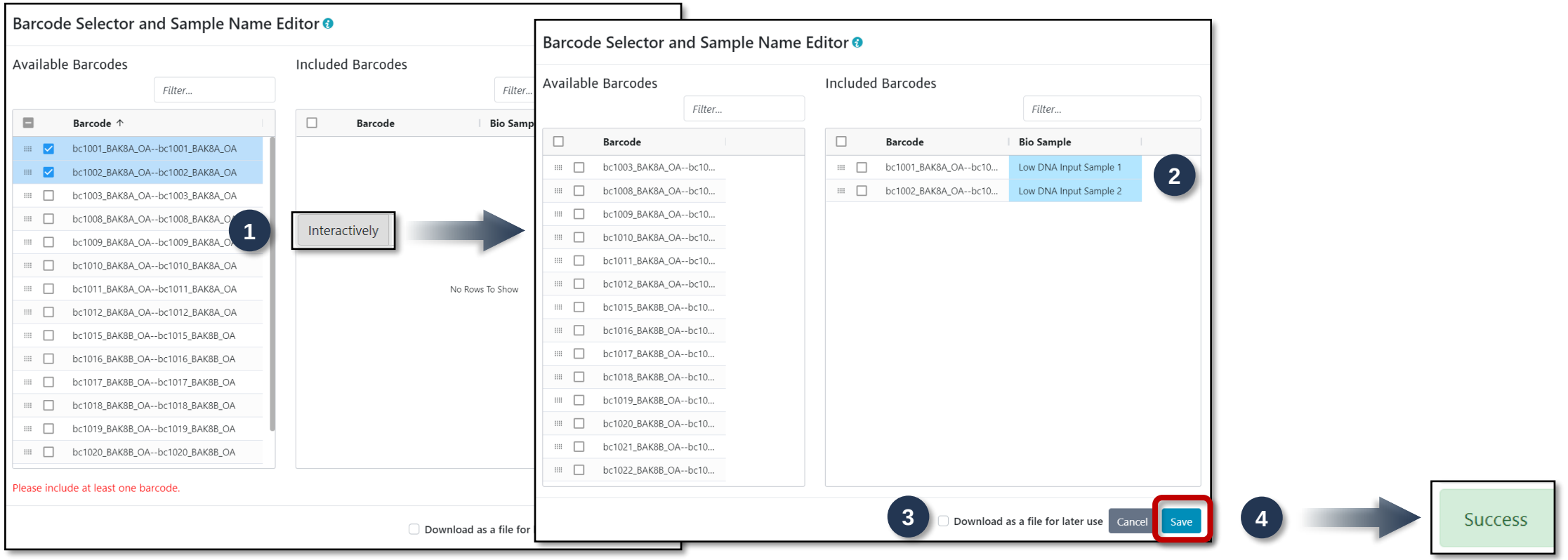
- Refer to **"Run Design" section in the SMRT Link User Guide** for further details



SMRT LINK RUN DESIGN SETUP PROCEDURE FOR AUTOMATED DEMULTIPLEXING OF POOLED LOW DNA INPUT LIBRARY SAMPLES (CONT.)

Interactive Method for Barcode Selection and Bio Sample Name Specification (SMRT Link v10.0 Only):

1. Click the **Interactively** button, then drag barcodes from the Available Barcodes column to the Included Barcodes column.
2. (Optional) Click a Bio Sample field to edit the Bio Sample Name associated with a barcode.
3. (Optional) Click Download as a file for later use.
4. Click Save to save the edited barcodes/bio sample names. You see Success on the line below, assuming the file is formatted correctly.



HiFi Sequencing Data Analysis Recommendations for *De Novo* Assembly Applications

HIFI SEQUENCING DATA ANALYSIS RECOMMENDATIONS FOR LOW DNA INPUT *DE NOVO* ASSEMBLY APPLICATIONS

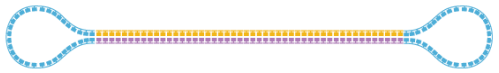
HiFi reads are compatible with third-party *de novo* assembly tools.

- Utilize SMRT Link to generate highly accurate and long single-molecule reads (**HiFi reads**) using the Circular Consensus Sequencing (CCS) analysis application or perform CCS analysis on-instrument using the Sequel IIe System
- Output data in standard file formats, (BAM and FASTA/Q) for seamless integration with downstream analysis tools
- **10- to 15-fold HiFi read coverage per haplotype** is recommended for Low DNA Input *de novo* assembly projects
- Can use PacBio's **SMRT Link Genome Assembly** analysis application (powered by IPA) or other third-party software for *de novo* assembly analysis using HiFi reads :
  - Hifiasm
  - HiCanu
- Contact PacBio Technical Support (support@pacb.com) or your local Bioinformatics Field Applications Scientist for additional information about data analysis recommendations

Low DNA Input Library Example Performance Data

EXAMPLE 1: NON-MULTIPLEXED BUTTERFLY LOW DNA INPUT LIBRARY (SINGLE-SAMPLE, SEQUEL II SYSTEM)

Sample Preparation and Sequencing Workflow



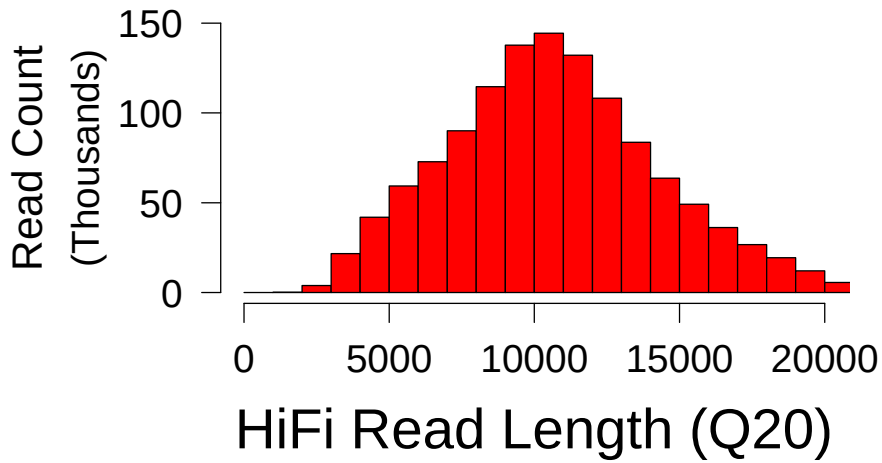
Single female Red Admiral butterfly (*Vanessa atalanta*) collected

HMW DNA (>40 kb) extraction using Qiagen MagAttract HMW Kit with modifications as described in Kingan et al. (2019)

Low DNA input SMRTbell library construction with 400 ng of input gDNA

Singleplex sequencing on the **Sequel II System** using a 30-hour collection time

A single SMRT Cell 8M produced **13 Gb of HiFi data**, or ~35-fold HiFi coverage of the ~350 Mb genome



Kingan, S.B. et al. (2020) Every species can be a model: Reference-quality PacBio genomes from single insects. PAG 2020 Poster.

EXAMPLE 1: NON-MULTIPLEXED BUTTERFLY LOW DNA INPUT LIBRARY (SINGLE-SAMPLE, SEQUEL II SYSTEM) (CONT.)

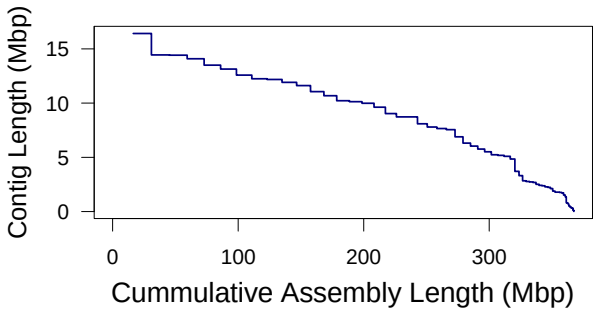
De Novo Assembly Performance Results

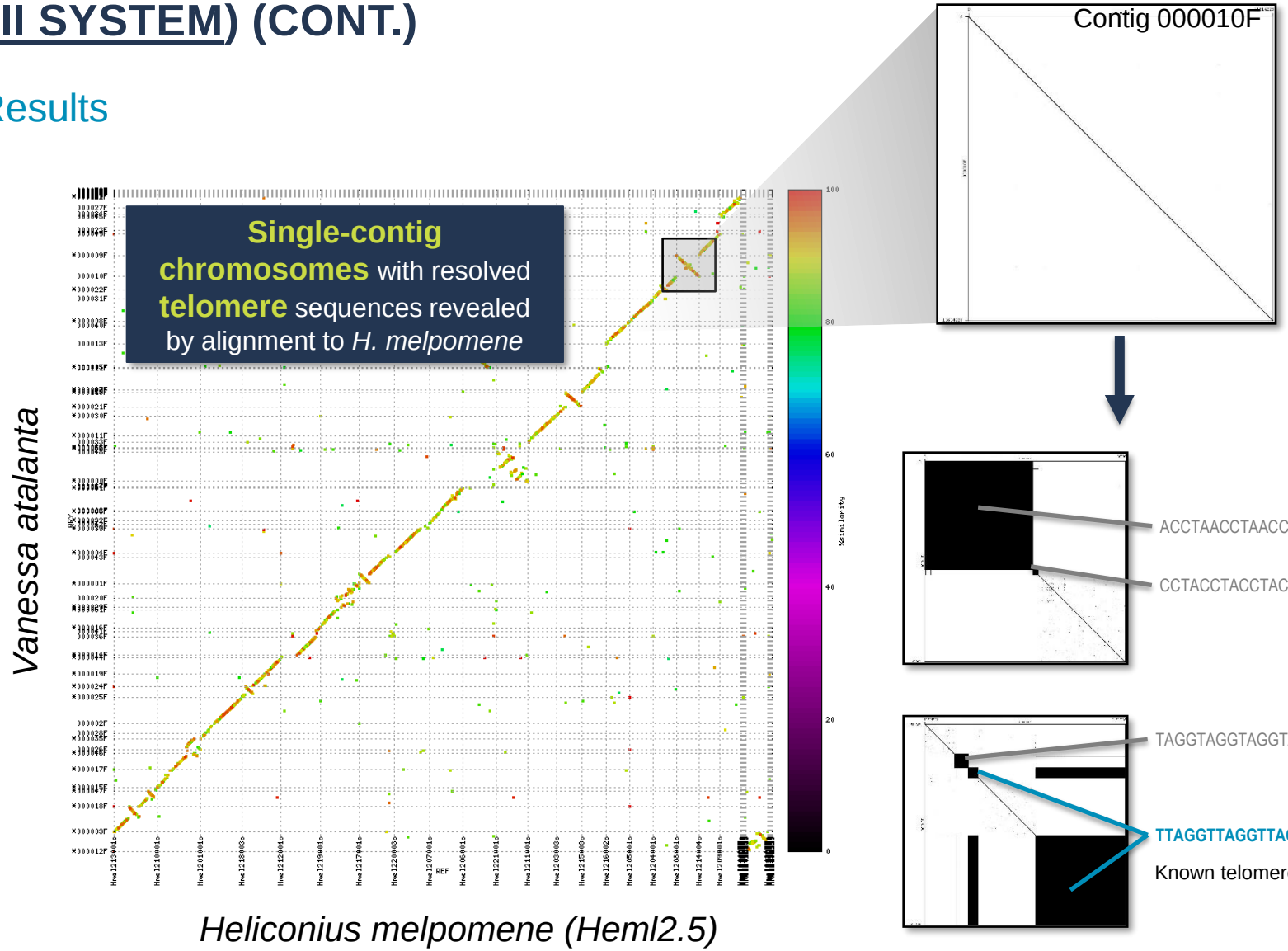
BUTTERFLY GENOME ASSEMBLY RESULTS.

METRIC	VALUE
Assembly Length	368 Mb
N Contigs	80
Contig N50	10.13 Mb
BUSCO Complete	99.1%
BUSCO Duplicate	0.3%

HiFi assembly using FALCON; polishing with Racon; haplotype deduplication with Purge Dups

FALCON + Purge-Dups

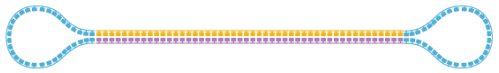




Kingan, S.B. et al. (2020) Every species can be a model: Reference-quality PacBio genomes from single insects. PAG 2020 Poster.

EXAMPLE 2: MULTIPLEXED MOSQUITO LOW DNA INPUT LIBRARY (2-PLEX SAMPLE, SEQUEL II SYSTEM)

Sample Preparation and Sequencing Workflow



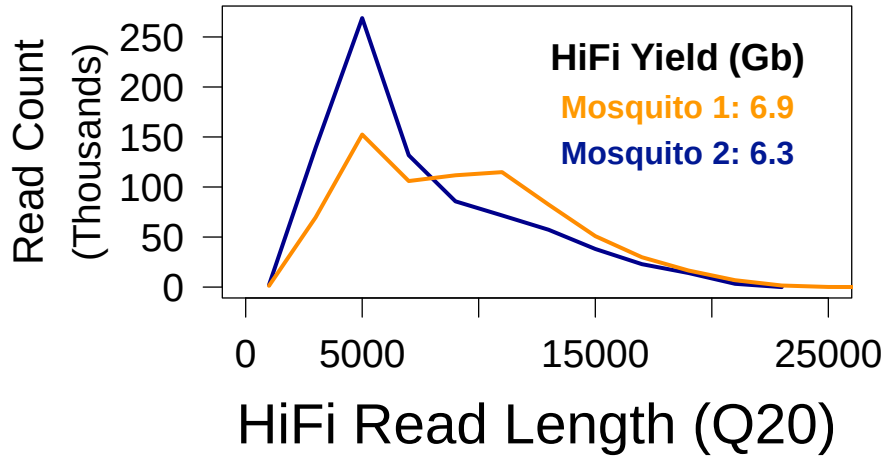
Two **individual** female mosquitos (*Anopheles coluzzii*) collected

HMW DNA (>40 kb) extraction using Qiagen MagAttract HMW Kit with modifications as described in Kingan et al. (2019)

Low DNA input SMRTbell library construction with 230 ng of input gDNA per mosquito sample

Multiplex 2 samples / SMRT Cell 8M for sequencing on the **Sequel II System** using a 30-hour collection time

A single SMRT Cell 8M produced **>6 Gb of HiFi data per sample**, or ~25-fold HiFi coverage of the ~250 Mb genome



Kingan, S.B. et al. (2020) Every species can be a model: Reference-quality PacBio genomes from single insects. PAG 2020 Poster.

EXAMPLE 2: MULTIPLEXED MOSQUITO LOW DNA INPUT LIBRARY (2-PLEX SAMPLE, SEQUEL II SYSTEM) (CONT.)

De Novo Assembly Performance Results

Assembly is Fast with HiFi Reads

MOSQUITO GENOME ASSEMBLY RESULTS.

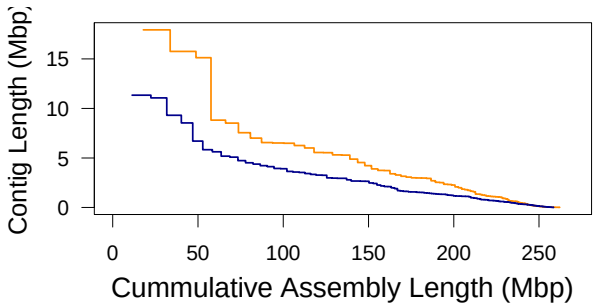
METRIC	MOSQUITO 1	MOSQUITO 2
Assembly Length	262 Mb	259 Mb
N Contigs	465	358
Contig N50	5.3 Mb	2.9 Mb
BUSCO Complete	98.8%	98.8%
BUSCO Duplicate	0.1%	0.3%

HiFi assembly using FALCON; polishing with Racon; haplotype deduplication with Purge Dups

MOSQUITO SAMPLE 1 SUBREADS ASSEMBLED AND COMPARED TO HIFI ASSEMBLY.

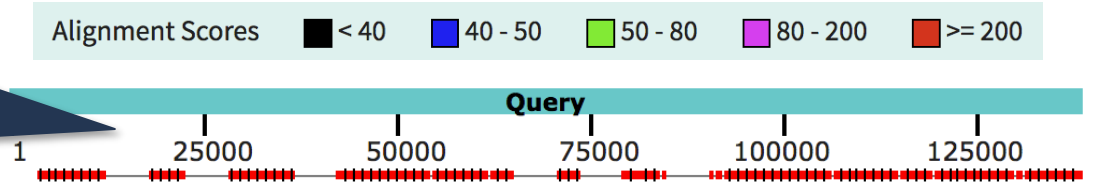
MOSQUITO 1	HIFI READ ASSEMBLY	LONG READ (CLR) ASSEMBLY
Coverage	25-Fold	40-Fold
N50 Read Length (N5)	11 kb (19 kb)	12 kb (22 kb)
Primary Assembly Length	262 Mb	243 Mb
Primary Contig N50	5.28 Mb	3.86 Mb
Primary Contigs	465	212
BUSCO	C:98.7%, D:0.1%, F:0.6%, M:0.7%	C:98.7, D:0.2% F:0.6%, M:0.7%
CPU Hours (Consensus + Accuracy)	1604	1947

FALCON + Purge-Dups



- 9 Mb of HiFi Read assembly does not map to Long Read assembly
- Primarily map to “UNKN” (96%) or sex chromosomes (3% Y, 1% X)
- A known satellite repeat (AgX367, L = 367 bp) maps across contig

HiFi Assemblies Capture Satellites and Other Repeats



000169F (L = 140 kb)

Kingan, S.B. et al. (2020) Every species can be a model: Reference-quality PacBio genomes from single insects. PAG 2020 Poster.

EXAMPLE 3: MULTIPLEXED MOTH LOW DNA INPUT LIBRARIES (2-PLEX SAMPLE, SEQUEL II SYSTEM)

Sample Preparation and Sequencing Workflow

Ten **individual** moth samples collected

↓

HMW DNA (>40 kb) extraction using Qiagen MagAttract HMW Kit with modifications as described in Kingan et al. (2019)

↓

Low DNA input SMRTbell library construction with 400 ng of input gDNA per moth sample

↓

Multiplex 2 samples / SMRT Cell 8M for sequencing on the **Sequel II System** using a 30-hour collection time

MOTH SPECIES	COMMON NAME	
Autographa gamma	Ssilver Y	
Cosmia trapezina	Dun-bar	
Craniophora ligustri	Coronet	
Endotricha flammealis	Rose-flounced tabby	
Lymantria monacha	Black-arched tussock	
Noctua fimbriata	Broad-bordered yellow underwing	
Notocelia uddmanniana	Bramble shoot	
Parapoynx stratiotata	Ringed china-mark	
Recurvaria leucatella	White-barred groundling	
Thyatira batis	Peach blossom	

EXAMPLE 3: MULTIPLEXED MOTH LOW DNA INPUT LIBRARIES (2-PLEX SAMPLE, SEQUEL II SYSTEM) (CONT.)

Assembly results using **Improved Phased Assembler (IPA)** show contig N50 ≥1 Mb for most samples analyzed.

De Novo Assembly Performance Results

MOTH GENOME ASSEMBLY RESULTS.

SAMPLE	ASSEMBLY SIZE (Mb)	# CONTIGS	CONTIG N50 (Mb)	BUSCO COMPLETE (ARTHROPODA)
Autographa gamma	368	79	12.01	99.2%
Cosmia trapezina	862	1924	0.88	97.5%
Craniophora ligustri	436	267	3.50	98.9%
Endotricha flammealis	489	489	1.92	99.2%
Lymantria monacha	912	301	5.77	99.2%
Noctua fimbriata	572	307	3.75	98.9%
Notocelia uddmanniana	814	809	2.07	98.3%
Parapoynx stratiotata	481	129	6.92	99.4%
Recurvaria leucatella	746	1247	0.98	98.2%
Thyatira batis	316	88	7.15	98.9%

HiFi assembly using Improved Phased Assembler (IPA); haplotype deduplication with Purge Dups

Technical Documentation & Applications Support Resources

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR LOW DNA INPUT LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS

Sample Preparation Literature

- Application Note: Considerations for Using the Low and Ultra-low DNA Input Workflows for Whole Genome Sequencing (PN 101-995-900)
- Procedure & Checklist - Procedure & Checklist - Preparing HiFi Libraries from Low DNA Input Using SMRTbell Express Template Prep Kit 2.0 (PN 101-730-400)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel System (PN 101-461-600)
- Quick Reference Card – Loading and Pre-extension Recommendations for the Sequel II/IIe Systems (PN 101-769-100)
- Overview – Sequel Systems Application Options and Sequencing Recommendations (PN 101-851-300)
- Application Consumable Bundles Purchasing Guide (PN PG100-051320)
- Technical Note: Preparing samples for PacBio whole genome sequencing for de novo assembly – Collection and storage (PN TN100-040518)
- Technical Note: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control (PN TN101-061920)
- Technical Overview: Low DNA Input Library Preparation Using SMRTbell Express Template Prep Kit 2.0 (PN 101-781-000)

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR LOW DNA INPUT LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Data Analysis Resources

- SMRT Analysis Barcoding Overview (v9.0) (PN 101-923-200)
  - Contains detailed information on barcoding experimental design options and describes QC metrics for evaluation of barcoding performance using SMRT Link
- PacBio Multiplexing Resources Website: https://www.pacb.com/products-and-services/analytical-software/multiplexing/
  - Barcoding Overview documents for different SMRT Link software versions
  - PacBio barcode sequence files (compressed FASTA) for use with Sequel, Sequel II and Sequel IIe Systems
  - Barcoded oligo ordering sheets

Example PacBio Data Sets

WHOLE GENOME SEQUENCING APPLICATION	DATASET	DATA TYPE	PACBIO SYSTEM
Assembly (Low DNA Input)	*Anopheles gambiae* (African malaria mosquito) – 2 plex	HiFi Reads	Sequel II System

TECHNICAL DOCUMENTATION & APPLICATIONS SUPPORT RESOURCES FOR LOW DNA INPUT LIBRARY PREPARATION, SEQUENCING & DATA ANALYSIS (CONT.)

Posters

- PacBio PAG 2020 Poster: Every species can be a model: Reference-quality PacBio genomes from single insects
- PacBio ABRF 2020 Poster: A low DNA input protocol for high-quality PacBio de novo genome assemblies
- PacBio AGBT 2020 Poster: New advances in SMRT Sequencing facilitate multiplexing for de novo and structural variant studies

Publications

- Kingan, S.B. et al. (2019) A high-quality de novo genome assembly from a single mosquito using PacBio sequencing. Genes. 10(1), 62.
- Kingan, S.B. et al. (2019) A high-quality genome assembly from a single, field-collected spotted lanternfly (Lycorma delicatula) using the PacBio Sequel II system. GigaScience 8(10):1 – 10.

Webinars

- PacBio Virtual Global Summit Presentation (2020): Anopheles and the journey from a reference genome to a thousand genomes to spatiotemporal genomic observatories. [Webinar Recording]
- PacBio Webinar (2020): No Organism Too Small: Build High-Quality Genome Assemblies of Small Organisms with HiFi Sequencing. [Webinar Recording]
- i5K Webinar (2019): High quality PacBio genomes from single insects: implications for vector research. [Webinar Slides – Part 1] [Webinar Slides – Part 2] [Webinar Recording]

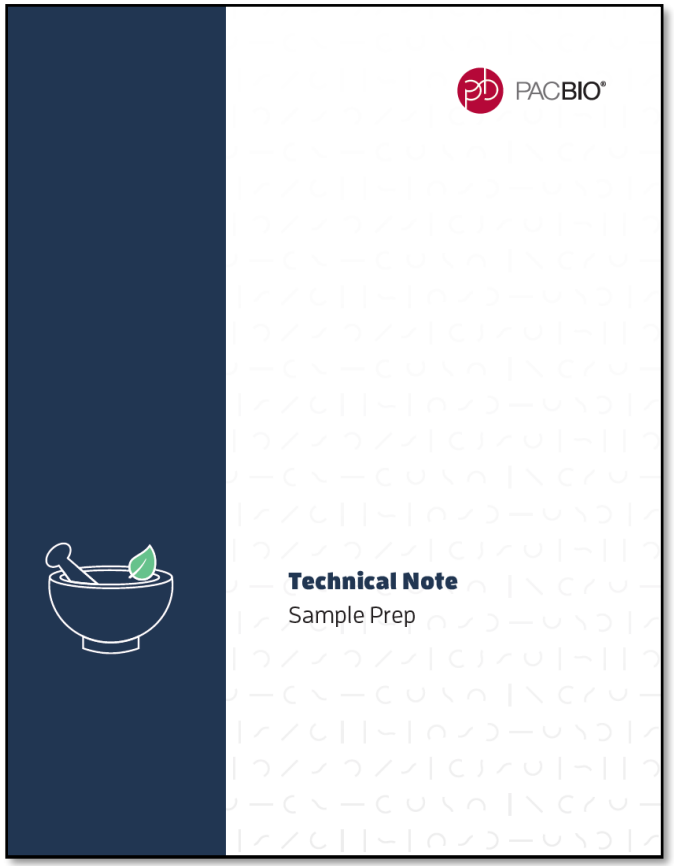
Appendix: General Recommendations for High-Molecular Weight gDNA QC and Handling for SMRTbell Library Construction

SAMPLE COLLECTION, PREPARATION, AND STORAGE FOR SMRT SEQUENCING WHOLE GENOME *DE NOVO* ASSEMBLY PROJECTS

To obtain the highest quality genomic DNA, it is important to start with sample types compatible with high molecular weight (HMW) DNA extraction methods

- **Technical Note: Sample Prep** (TN100-040518) provides general guidance on biological sample collection, preparation, and storage across a range of commonly encountered sample types used for SMRT Sequencing whole genome projects
- Includes sample and storage recommendations for:
  - Vertebrates - mammals, birds, fish, amphibians, reptiles
  - Invertebrates - marine, terrestrial
  - Arthropods - insects, crustaceans
  - Fungi - microorganisms, mushrooms, algae*
  - Plants - broad leaf plants, grasses

  *Algae is included with fungi due to similar growth and storage conditions
- This technical note also Includes additional considerations for planning HMW DNA isolation



Technical Note (TN100-040518): Preparing samples for PacBio whole genome sequencing for *de novo* assembly – Collection and storage

DNA EXTRACTION AND QC FOR SMRT SEQUENCING WHOLE GENOME *DE NOVO* ASSEMBLY PROJECTS

Starting with high-quality, high molecular weight (HMW) genomic DNA (gDNA) will result in longer libraries and better performance during sequencing.

- **Technical Note: DNA Prep** (TN101-061920) is intended to give recommendations, tips and tricks for the extraction of genomic DNA, as well as assessing and preserving the quality and size of your DNA sample to be used for PacBio HiFi sequencing for *de novo* assembly
- Topics covered include:
  - DNA Extraction
    - Commercially available kits across a wide variety of input sample types
    - Resource for alternative DNA extraction methods
  - DNA Quality Control (QC)
    - DNA quantification, purity, size, and damage
    - Use of nucleic acid stabilizers
    - DNA storage and shipping
  - Best Practices for DNA Extraction for PacBio Sequencing
- This technical note also includes an example dataset for a California Redwood tree DNA sample that was isolated using a commercial DNA extraction kit

Technical Note TN101-061920: Preparing DNA for PacBio HiFi sequencing – Extraction and quality control

DNA SAMPLE PREPARATION ONLINE RESOURCE

Literature resource for sample collection and DNA extraction protocol references

The listing below is a collection of publications by the scientific community describing extraction protocols for high-molecular weight DNA followed by PacBio sequencing. When possible, the links point directly to the methods section (or supplementary information).

Animals | Plants | Fungi | Protists

If you have protocols you would like to share, or have questions about DNA extraction for PacBio sequencing, contact ExtractDNA@pacb.com.

Animals

- Invertebrates
  - Panova2016 – DNA extraction protocols for whole-genome sequencing in marine organisms
  - *microinvertebrates*
    - Laumer2020 – protocols for diverse meiofauna species, including *C. elegans* & *Castrella truncata* (SMRT Leide… confer… presentation)
  - *arthropods*
    - arachnids
      - Guerrero2019 – The Pacific Biosciences de novo assembled genome dataset from a parthenogenetic New Zealand wild population of the longhorned tick, *Haemaphysalis longicornis* Neumann, 1901
      - Liu2019 – DNA Methylation Patterns in the Social Spider, *Stegodyphus dumicola*
      - Burgess2018 – Draft genome assembly of the sheep scab mite, *Psoroptes ovis*
      - Randall2018 – The draft genome assembly of *Dermatophagoides pteronyssinus* supports identification of novel allergen isoforms in *Dermatophagoides* species

…ins by ultra-long single

…ous, Annotated Genome

…s the Sex Chromosome

…phnia

Methods

Sample collection

A female yellowbelly pufferfish (Fig. 2), reared in the fish breeding centre of Fujian Normal University in Fuzhou City of Fujian Province was used for genome sequencing and assembly. Fresh white muscle, eye, skin, gonad, gut, liver, kidney, blood, gall bladder and air bladder tissues were collected and quickly frozen in liquid nitrogen for one hour. White muscle tissues were used for DNA sequencing for genome assembly, while all tissues were used for transcriptome sequencing.

Fig. 2

A picture of the yellowbelly pufferfish used in the genome sequencing and assembly.

DNA and RNA sequencing

Genomic DNA from white muscle tissue was extracted using the

Plants

…t genomic DNA for long-read sequencing of single molecules

…ce Genome With Single-Molecule Sequencing Uncovers a Recent Burst of … Genes

…ssembly reveals the structure of the *Arabidopsis thaliana* Nd-1 genome and its gene set

…sive, high-…

…ng and se…

…ect run at PacBio (using the Circulomics Nanobind Plant Nuclei Big DNA Kit)

…olly pine mega-genome using long-read single-molecule sequencing

…ce for the Fast-Growing Microalga *Picochlorum celeri*

Undaria pinnatifida: Chromosome-Level Assembly Using PacBio and Hi-C

…e suggests convergent functions of homeobox genes in algae and land plants (algae)

- Panova2016 – DNA extraction protocols for whole-genome sequencing in marine organisms (algae)

Fungi

- Faure2019 – Long-Read Genome Sequence of the Sugar Beet Rhizosphere Mycoparasite *Pythium oligandrum*
- Nagappan2018 – Improved nucleic acid extraction protocols for *Ganoderma boninense*, *G. miniatocinctum* and *G. tornatu*
- Schwessinger2017 – Extraction of high molecular weight DNA from fungal rust spores for long read sequencing
- Solomon2016 – Robust and effective methodologies for cryopreservation and DNA extraction from anaerobic gut fungi
- Sonnenberg2016 – A detailed analysis of the recombination landscape of the button mushroom *Agaricus bisporus var. bisporus*

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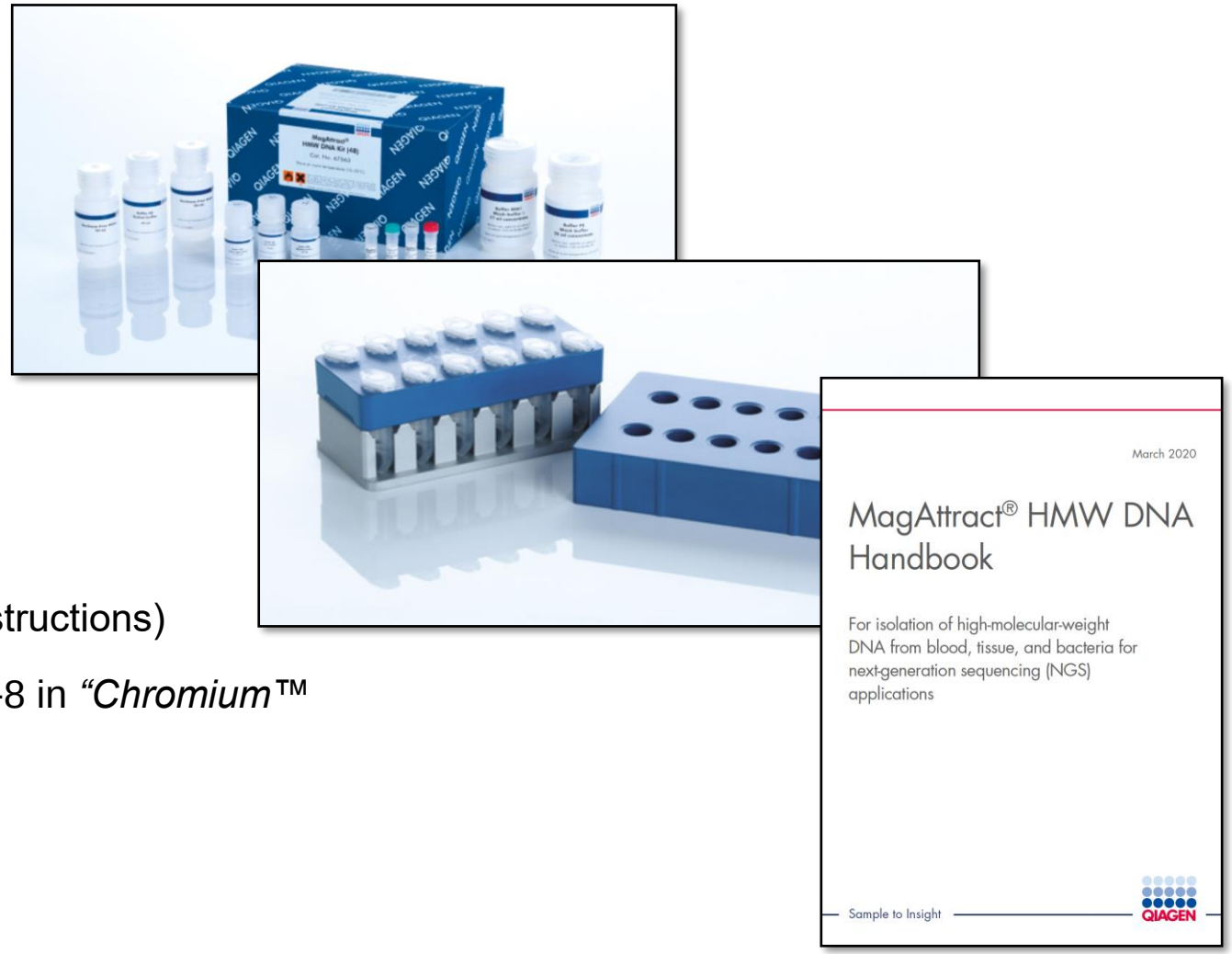
PacBio does not assume responsibilities/guarantees for these external publications/protocols, but we are happy to help as best as we can to guide / connect. Please contact **ExtractDNA@pacb.com** for more discussions around your particular species & sequencing project!

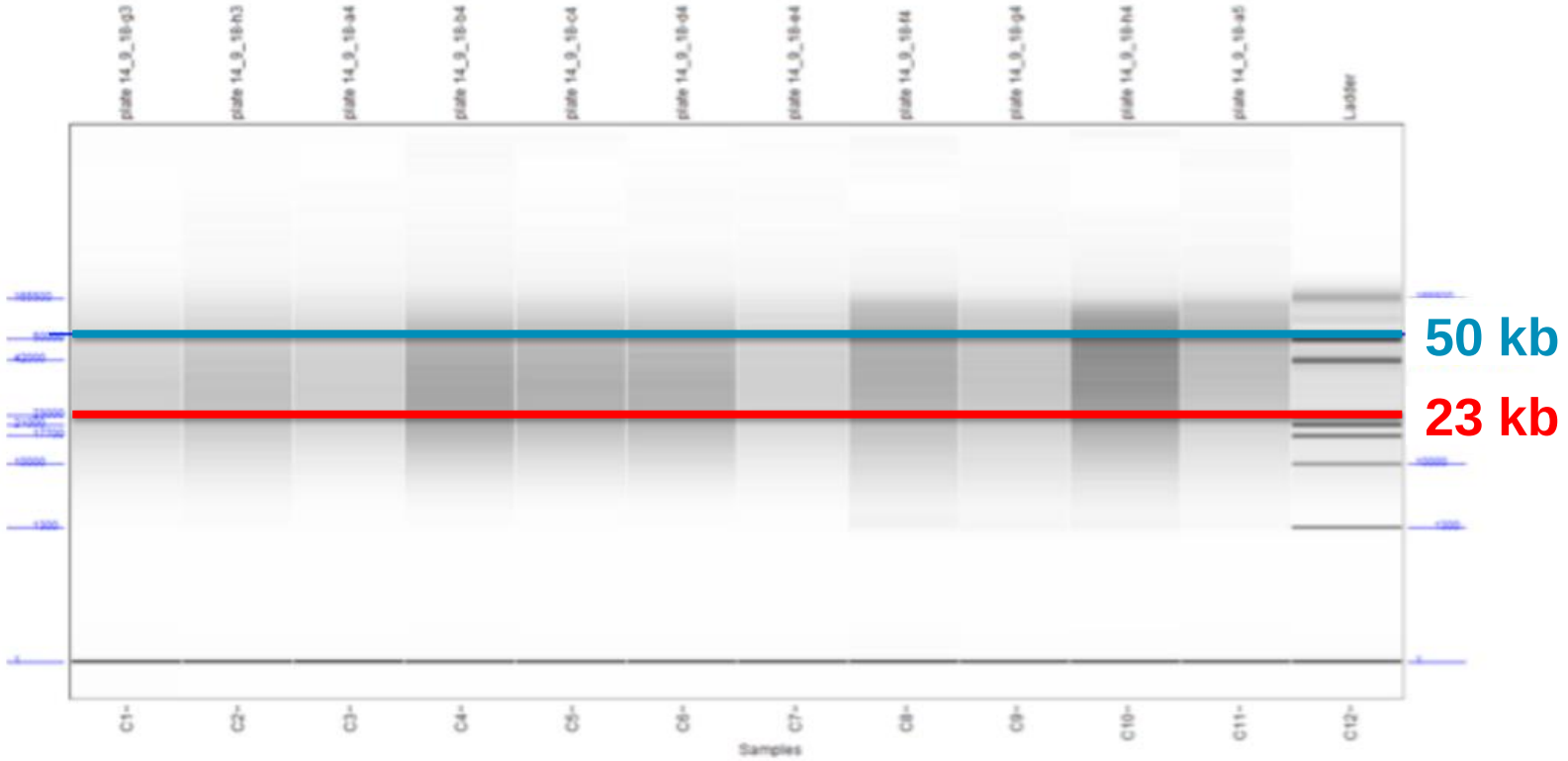
RECOMMENDED EXTRACTION KIT FOR ISOLATION OF HMW GENOMIC DNA FOR LOW DNA INPUT LIBRARY PREPARATION

- **QIAGEN MagAttract HMW DNA Kit**
  - Cat. No/ID: 67563
- **MagAttract Magnetic Rack**
  - Cat. No/ID: 19606
- **MagAttract Protocol Versions**
  - "Standard" protocol (as per QIAGEN instructions)
  - 10X Genomics-modified protocol (pp. 6-8 in *"Chromium™ Genome Reagent Kits v2 User Guide"*)



http://i5k.github.io/webinar_slides/i5k_webinar_PacBio_Lawniczak_Mar06_2019.pdf

EXAMPLE RESULTS USING “STANDARD” QIAGEN MAG ATTRACT EXTRACTION PROTOCOL FOR MOSQUITO GENOMIC DNA EXTRACTION



- Figure shows typical size distribution profiles obtained for gDNA samples extracted from fresh or freshly killed and stored at -80°C mosquitos
- Recommend preparing multiple DNA extractions for evaluation of prior to library construction (**Note:** Majority of gDNA must be >30 kb and <100 kb)
- Typically obtain ~200 ng of HMW DNA per mosquito sample

http://i5k.github.io/webinar_slides/i5k_webinar_PacBio_Lawniczak_Mar06_2019.pdf

METHODS FOR EVALUATION OF GENOMIC DNA QUALITY

Starting with high-quality genomic DNA will result in longer libraries and better *de novo* assembly performance

- Input genomic DNA must be carefully QC'd to assess integrity
- PFGE/FIGE or Femto Pulse sizing tool is highly recommended
- High-quality, high-molecular weight DNA → longer read lengths
- Low-quality, degraded/damaged DNA → shorter read lengths, lower library synthesis yields (dependent on size selection method and parameter settings employed)
- DNA purity can be determined by using a NanoDrop instrument or other spectrophotometer device
- PacBio highly recommends using the Qubit High Sensitivity fluorometric assay for accurate dsDNA quantitation

A. Genomic DNA Sizing Characterization

Recommended methods for determining gDNA size distribution:

PippinPulse System (Sage Science)

Resolves up to ~80 kb
Requires ≥50 ng of sample
~16-hour analysis time

http://www.sagescience.com/products/pippin-pulse/

Femto Pulse System (Agilent Technologies)

Highly Recommended

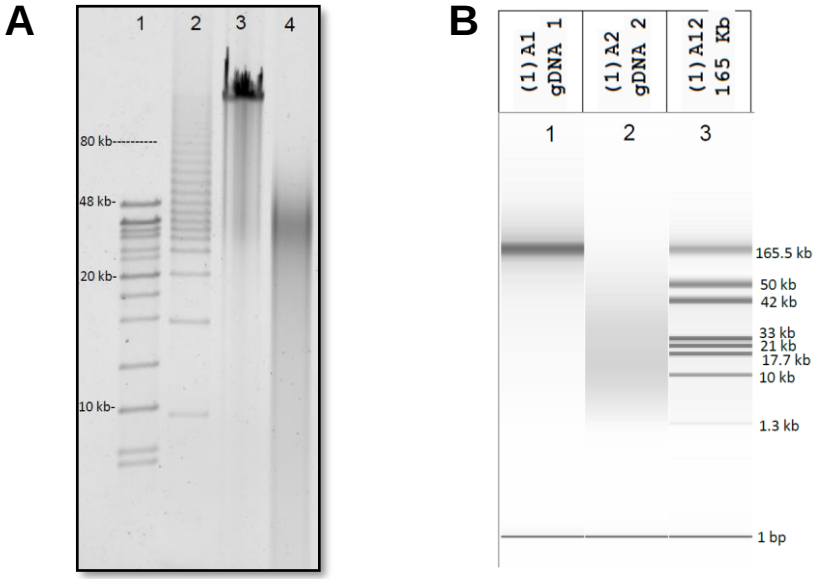
Resolves up to ~165 kb
Requires <1 ng of sample
<1.5-hour analysis time

https://www.agilent.com/en/product/automated-electrophoresis/femto-pulse-systems

CHEF Mapper XA System (Bio-Rad)

Resolves up to ~10 Mb
Requires ≥100 ng of sample
~16-h analysis time

http://www.bio-rad.com/en-us/category/pulsed-field-gel-electrophoresis-systems



Lane 1: 8-48 kb Ladder (Bio-Rad)
Lane 2: 5 kb Ladder (Bio-Rad)
Lane 3: HMW gDNA
Lane 4: Degraded gDNA

Lane 1: High MW gDNA
Lane 2: Degraded gDNA
Lane 3: 165 kb Ladder

Evaluation of gDNA quality using A) Bio-Rad CHEF Mapper System and B) Femto Pulse System. Lanes 3A and 1B are examples of high quality, high-molecular weight genomic DNA. Lanes 4A and 2B are examples of degraded gDNA.

B. DNA Purity Determination

- DNA purity can be determined by using a **NanoDrop** instrument or other spectrophotometers
- For ultrapure gDNA, A260/280 ratio is typically between ~1.8 - 2.0 and A260/230 ratio is ≥2.0
- If A260/280 and A260/230 readings are out of the range specified above, PacBio recommends performing an AMPure PB bead purification step followed by re-assessment of quantity and purity of the gDNA sample

260/280 Ratio

- A low A260/A280 ratio may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. Sometimes it may be caused by a very low concentration of nucleic acid.
- High 260/280 ratios are not indicative of an issue

260/230 Ratio

- A low A260/A230 ratio may be the result of:
  - Carbohydrate carryover (often a problem with plants)
  - Residual phenol from nucleic acid extraction
  - Residual guanidine (often used in column-based kits)
  - Glycogen used for precipitation
- A high A260/A230 ratio may be the result of:
  - Making a blank measurement on a dirty pedestal of a Nanodrop instrument
  - Using an inappropriate solution for the blank measurement

C. DNA Quantification

- Accurate quantitation of DNA concentration is critical for PacBio template preparation procedures.
  - Specifically, it is critical to determine the concentration of the double-stranded DNA, since only double-stranded DNA will be converted into sequencing templates.
- PacBio highly recommends using a **Qubit fluorometer** tool and **Qubit dsDNA High Sensitivity (HS) Assay Kit** (Thermo Fisher Scientific) for routine DNA quantitation during SMRTbell library construction.
- When assessing gDNA QC, PacBio recommends using both fluorometric and spectrophotometric methods – for example, using both the Qubit and NanoDrop instruments
  - If the sample is pure gDNA, free of any RNA contaminants and other small molecules, the two methods should converge to similar DNA concentration measurement values
- If the measured NanoDrop concentration is significantly different (>50%) from the Qubit measurement, PacBio recommends doing an AMPure PB bead purification step (as specified by your chosen library preparation protocol), followed by a re-measurement with both methods. Typically, a single AMPure PB bead purification step resolves the discrepancy.
  - If the agreement does not improve after three rounds of AMPure PB bead purification, try using either a commercial kit, isopropanol precipitation, or a new DNA extraction method to obtain a cleaner DNA sample..

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