PacBio Americas User Group Meeting Sample Prep Workshop Breakout Session:
Short Insert Library Prep & Amplicon Sequencing

June.27.2017 / http://programs.pacificbiosciences.com/l/1652/2017-03-25/3sn5p2

AGENDA

Introduction

- Amplicon Sequencing Applications
- Technical Resources for Amplicon Sequencing
- Recommendations for Preparing High-quality Amplicons for SMRTbell library construction
- Recommended Amplicon Sequencing Conditions (PacBio RS II / Sequel)
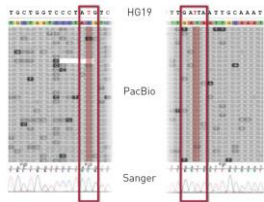- Where to Find SMRT Resources

Customer Presentations

- **"Rescuing Ebola Makona Using Reverse Genetics and SMRT Sequencing"**

  David Kimmel, United States Army Medical Research Institute of Infectious Diseases (USAMRIID)
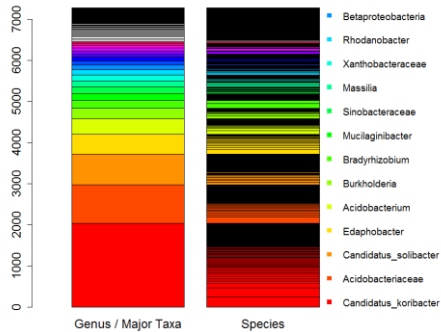
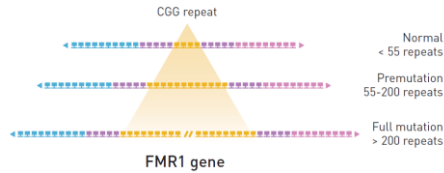## Q&A and Open Discussion

AMPLICON SEQUENCING APPLICATIONS

Highly Accurate SNP Detection and Validation

- Highly accurate SNP validation for any genomic region reduces false positives and false negatives.
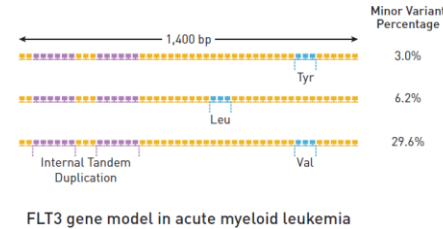
Repeat Expansion Analysis in Low Complexity Regions

- Span extreme CGG repeats and AT-rich regions with minimal bias, over hundreds to thousands of bases

Compound Mutations and Haplotype Phasing

- Study linked mutations hundreds, even thousands, of bases apart

- Differentiate polyclonal from compound mutations

True HLA Allelic Diversity Assessment

- Generate highly accurate consensus sequences spanning full-length HLA genes to obtain directly phased, high-resolution HLA types without imputation

Characterize Metagenomic Communities

- Resolve community composition and phylogeny using full-length 16S rRNA sequences

- Discover novel genes and gene clusters from longer reads and assembled contigs

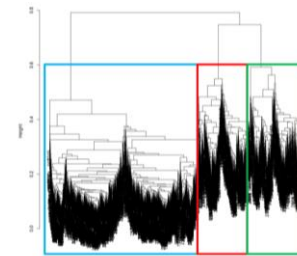### Detect Minor Variants in Complex Mixtures

- Exquisitely sensitive and specific analysis of mixed populations (MV detection to 1% frequency)

Resolve Viral Populations

- Deconvolute complex mixtures of unique haplotypes

- Track evolution and phylogeny of viral populations

TECHNICAL RESOURCES FOR AMPLICON SMRTBELL LIBRARY SAMPLE PREPARATION AND SEQUENCING

User Bulletins

*User Bulletin for PacBio RS II and Sequel Systems: Centrifuge Tube and Pipet Tip Recommendations (*NEW) (May 2017)*
- PacBio advises against the use of Axygen MAXYMum Recovery™ tubes and pipet tips. Please discontinue use of these products immediately. PacBio recommends alternatives in the User Bulletin.
- http://www.pacb.com/wp-content/uploads/User-Bulletin-Centrifuge-Tube-and-Pipet-Tip-Recommendations.pdf

*Field Advisory for Sequel System: Securing Sequel Pipet Tip Rack (*NEW) (May 2017)*
- PacBio recommends a simple procedure to ensure that the Sequel Pipet Tip rack is firmly affixed to the tip box.
- http://www.pacb.com/wp-content/uploads/Field-Advisory-Notice-Securing-Sequel-Pipet-Tip-Rack.pdf

*User Bulletin for Sequel System: Heat Seal Advisory (Adhesive Seal Warning) (*NEW) (May 2017)*
- PacBio advises against the use of adhesive foils and recommends the use of Sequel Sample Plate Foil.
- http://www.pacb.com/wp-content/uploads/User-Bulletin-Heat-Seal-Advisory-Adhesive-Seal-Warning.pdf

*User Bulletin for Sequel System: Barcode Scanning of Sequel Sequencing Kit 2.0 (*NEW) (May 2017)*
- PacBio is providing clarity on which barcode to scan to ensure the Sequel System has the correct information and that all the consumables are compatible.
- http://www.pacb.com/wp-content/uploads/User-Bulletin-Barcode-Scanning-of-Sequel-Sequencing-Kit-2.0.pdf
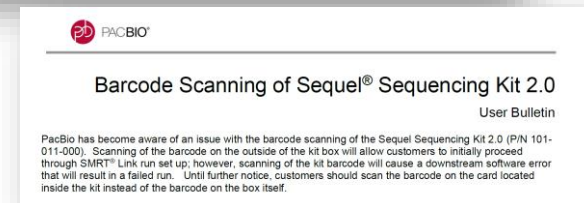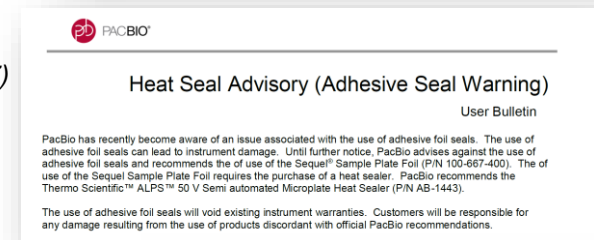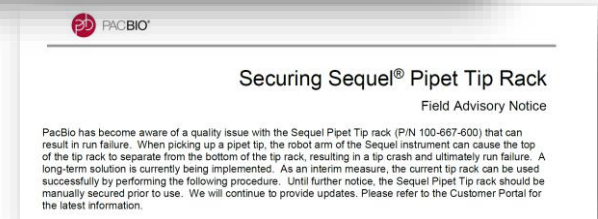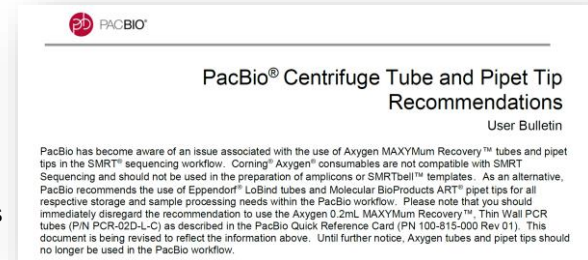
Find all protocols at http://www.pacb.com/support/documentation/

Amplicon SMRTbell Library Preparation Protocols

Procedure & Checklist – Amplicon Template Preparation and Sequencing

- http://www.pacb.com/wp-content/uploads/Procedure-Checklist-Amplicon-Template-Preparation-and-Sequencing.pdf

Shared Protocol – ≤250 bp Amplicon Library Preparation and Sequencing

- http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-250-bp-Amplicon-Library-Preparation-and-Sequencing.pdf

Multiplexed Amplicon SMRTbell Library Preparation Protocols

Procedure & Checklist – Preparing SMRTbell™ Libraries using PacBio® Barcoded Universal Primers for Multiplex SMRT® Sequencing

- http://www.pacb.com/wp-content/uploads/2015/09/Procedure-and-Checklist-Preparing-SMRTbell-Libraries-PacB-Barcoded-Universal-Primers.pdf

Procedure & Checklist - Preparing Amplicon Libraries using PacBio® Barcoded Adapters for Multiplex SMRT® Sequencing

- http://www.pacb.com/wp-content/uploads/2015/09/Procedure-Checklist-Preparing-SMRTbell-Libraries-using-PacBio-Barcoded-Adapters-for-Multiplex-SMRT-Sequencing.pdf

User Bulletin – Barcode Plate Mapping

- http://www.pacb.com/wp-content/uploads/2015/09/User-Bulletin-Barcode-Plate-Mapping.pdf

Full-Length 16S Amplicon SMRTbell Library Preparation Protocol

Unsupported Protocol – Unsupported Protocol – Full-Length 16S Amplification, SMRTbell Library Preparation and Sequencing

- http://www.pacb.com/wp-content/uploads/Unsupported-Full-Length-16S-Amplification-SMRTbell-LibraryPreparation-and-Sequencing.pdf

Product Notes

Product Note: Multiplexing Amplicons Up To 10 kb Using Barcoded Adapters and Barcoded Universal Primers

- http://www.pacb.com/wp-content/uploads/2015/09/ProductNote-Barcoded-Adapters-Barcoded-Universal-Primers.pdf

Genomic DNA Cleanup

Unsupported Protocol – High Salt Phenol Chloroform Cleanup

- http://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf

SMRTbell Library Cleanup

Unsupported Protocol – Purification of Contaminated SMRTbell™ Library Using Magnetic Bead Capture

- http://www.pacb.com/wp-content/uploads/2015/09/Purifying-Contaminated-SMRTbell-Libraries-Using-MagBeads-052013.pdf

Find all protocols at http://www.pacb.com/support/documentation/

GENERAL WORKFLOW FOR AMPLICON SAMPLE PREPARATION AND SEQUENCING

Find all protocols at http://www.pacb.com/support/documentation/

SAMPLE COLLECTION AND NUCLEIC ACID EXTRACTION

| SAMPLE COLLECTION | NUCLEIC ACID EXTRACTION | AMPLIFICATION | QC AMPLICON | SMRTBELL CONSTRUCTION/ SEQUENCING | DATA ANALYSIS |

- Environmental
- Plants/animals
- Human
- Tissues
- Cultures
- FFPE

- RNA or DNA

General Guidance:

- Store/ transport specimens in proper buffer ,additives and temperature to preserve the nucleic acid content
- Reduce DNA damages: Avoid high temperature, UV light, aliquot samples to minimize freeze/thaw cycles
- Remove contaminants: polysaccharides, proteins……
- High-molecular weight genomic DNA

AMPLICON GENERATION

| SAMPLE COLLECTION | NUCLEIC ACID EXTRACTION | AMPLIFICATION | QC AMPLICON | SMRTBELL CONSTRUCTION/ SEQUENCING | DATA ANALYSIS |

EPIGENETIC SIGNATURES ARE REMOVED!

- Targeted PCR: 16S, HLA, viral genes/genomes
- Target capture enrichment: NimbleGen SeqCap, IDT, Agilent SureSelect
- Targeted or whole transcriptome: Iso-Seq
- Whole genome amplification (WGA)

GENERATING HIGH-QUALITY PCR PRODUCTS

SAMPLE COLLECTION	NUCLEIC ACID EXTRACTION	AMPLIFICATION	QC AMPLICON	SMRTBELL CONSTRUCTION/ SEQUENCING	DATA ANALYSIS

- Begin with high-quality nucleic acids
  - Use fresh nucleic acids as templates in amplification reactions.
  - Store / freeze at high concentrations in appropriately-buffered solutions.
  - To minimize degradation and possible contamination, sub-aliquot extracts into smaller volumes for storage. For DNA samples, DNAStable® Plus (Biomatrica) may be used to help preserve extracted DNA.
  - Do DNA repair if damage to input DNA is suspected.
  - Do not expose DNA to intercalating fluorescent dyes or ultraviolet radiation. SYBR dyes are not DNA damaging, but do avoid ethidium bromide.
- Use PCR reagents and conditions likely to generate clean, undamaged, and non-chimeric amplicons
  - High-quality primers
  - High fidelity polymerase
  - Minimize high temperature time and cycle numbers
  - Ensure extension time is long enough to complete template synthesis to avoid chimera generation
- Multiplexing Options
  - Amplicon-specific barcoded PCR primers
  - PacBio® Barcoded Universal Primers kit
  - PacBio® Barcoded Adapters Kit

AMPLICON QC

- Visual check: Foamy? Insoluble material? Cloudy?.....

- Agarose gel/Bioanalyzer: RNA, primers, non-specific bands

- Spectrophotometer: Spectral profile shifts
    - Common chemicals, phenol, guanidine, DMSO, etc. can cause spectral shifts
    - 260 nm/280 nm ~1.8-2.0
    - 260 nm/230 nm: ~2.0-2.2

- Options to follow
    - Clean up amplicons before going into SMRTbell library prep
    - Perform size selection to clean up non-specific products

Shifts in Spectral Profile
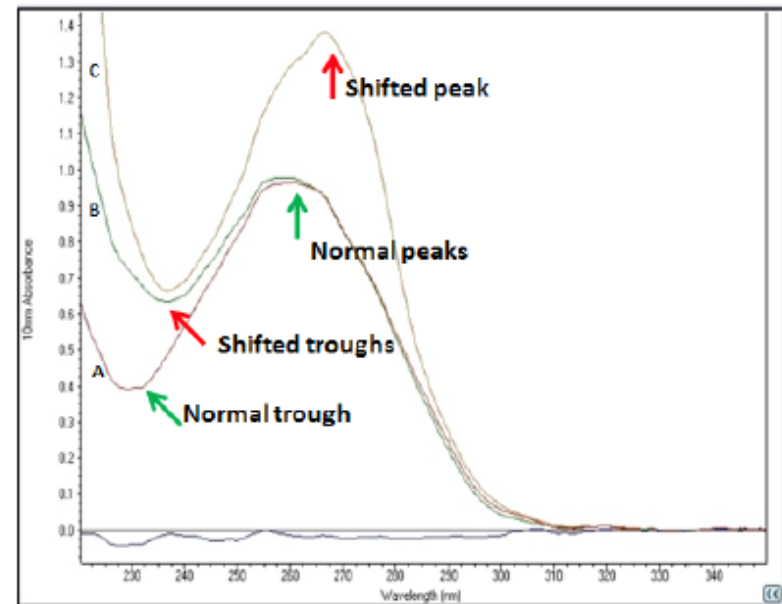
FIGURE 2. Spectra of purified DNA without contamination (A), and of the same DNA sample contaminated with guanidine (B) and phenol (C). www.nanodrop.com

LIBRARY CONSTRUCTION AND SEQUENCING

| SAMPLE COLLECTION | NUCLEIC ACID EXTRACTION | AMPLIFICATION | QC AMPLICON | SMRTBELL CONSTRUCTION/ SEQUENCING | DATA ANALYSIS |

Standard Protocol for Amplicons 250 bp – 10 kb or Larger

Procedure & Checklist - Amplicon Template Preparation and Sequencing

Before You Begin

To perform this procedure, you must have the following PacBio® products:

- SMRTbell™ Template Prep Kit
- DNA/Polymerase Binding Kit
- MagBead Kit for amplicons ≥1 kb
- DNA Sequencing Reagent
- DNA Internal Control Complex
- SMRT® Cells
- AMPure® PB beads

The PacBio System can be used to generate highly accurate sequences from amplicons ranging from several hundred bases to 10 kb or larger. Unlike sheared genomic DNA (gDNA), which is comprised of DNA fragments spanning a range of lengths, PCR products from one reaction are typically the same or similar lengths. This document describes methods for preparing PCR-amplified DNA for sequencing on the PacBio System.

Protocol for Full-Length 16 Amplicon Sequencing

Unsupported Protocol
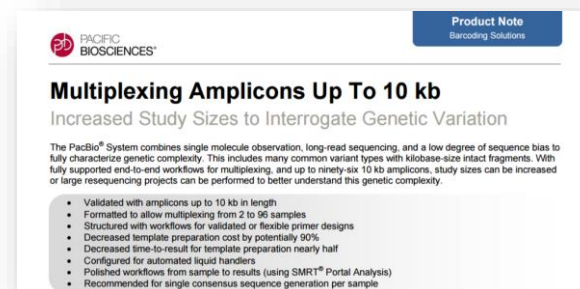
Please note: the shared protocols described herein may not have been validated by Pacific Biosciences and are provided as-is and without any warranty. Use of these protocols is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio® system. If any of these protocols are to be used in a production environment, it is the responsibility of the end user to perform the required validation.

Full-Length 16S Amplification, SMRTbell™ Library Preparation and Sequencing

This document contains protocols for amplification and sequencing of the entire 16S gene from bacterial DNA isolated from metagenomic samples. Tests with mock community samples produced discrete 16S amplicons with adequate yield for library prep and SMRT sequencing. Data analysis showed good representation of community members in the samples, with low rates of chimerism.

Alternative Protocol for Amplicons <250 bp

PacBio SampleNet - Shared Protocol

Please note: the shared protocols described herein may not have been validated by Pacific Biosciences and are provided as-is and without any warranty. Use of these protocols is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio® System. If any of these protocols are to be used in a production environment, it is the responsibility of the end user to perform the required validation.

250 bp Amplicon Library Preparation and Sequencing

Before You Begin

To perform this procedure, you must have the PacBio® Template Prep Kit.

This procedure is optimized for SMRTbell™ template preparation from PCR amplicons of less than 250 bp. Although sheared DNA can be used in this procedure, we find that yields are significantly lower than when starting with PCR products.

Protocols for Multiplexed Amplicon Sequencing

Procedure & Checklist - Preparing Amplicon Libraries using PacBio® Barcoded Adapters for Multiplex SMRT® Sequencing

Before You Begin

This document describes methods for generating Amplicon libraries using PacBio barcoded adapters.

The procedure is a modification to the standard library preparation procedure in that samples must first go through a single tube End-Repair and Ligation reaction. After ligation, samples (depending on the desired multi-plex level) are pooled in equimolar quantities for DNA damage repair, followed by treatment with Exo III and VII.

Procedure & Checklist - Preparing SMRTbell™ Libraries using PacBio® Barcoded Universal Primers for Multiplex SMRT® Sequencing

Before You Begin

This document describes methods for generating barcoded PCR products using PacBio Barcoded Universal Primers (BUP) and subsequently constructed to SMRTbell libraries.

The procedure provides recommendations for amplifying targets using primers tailed with two different universal sequences. The amplified products are further amplified using barcoded universal primers. The barcoded PCR products are pooled for SMRTbell library construction and subsequently sequenced on the PacBio system.

Unsupported Protocol

Please note: the shared protocols described herein may not have been validated by Pacific Biosciences and are provided as-is and without any warranty. Use of these protocols is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio® RS II system. If any of these protocols are to be used in a production environment, it is the responsibility of the end user to perform the required validation.
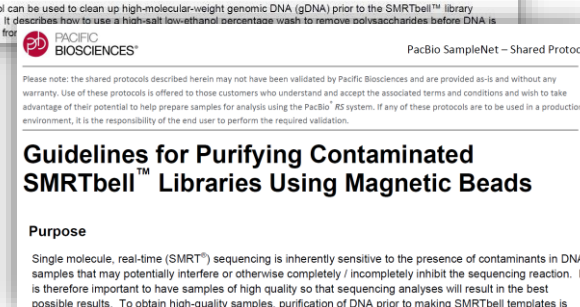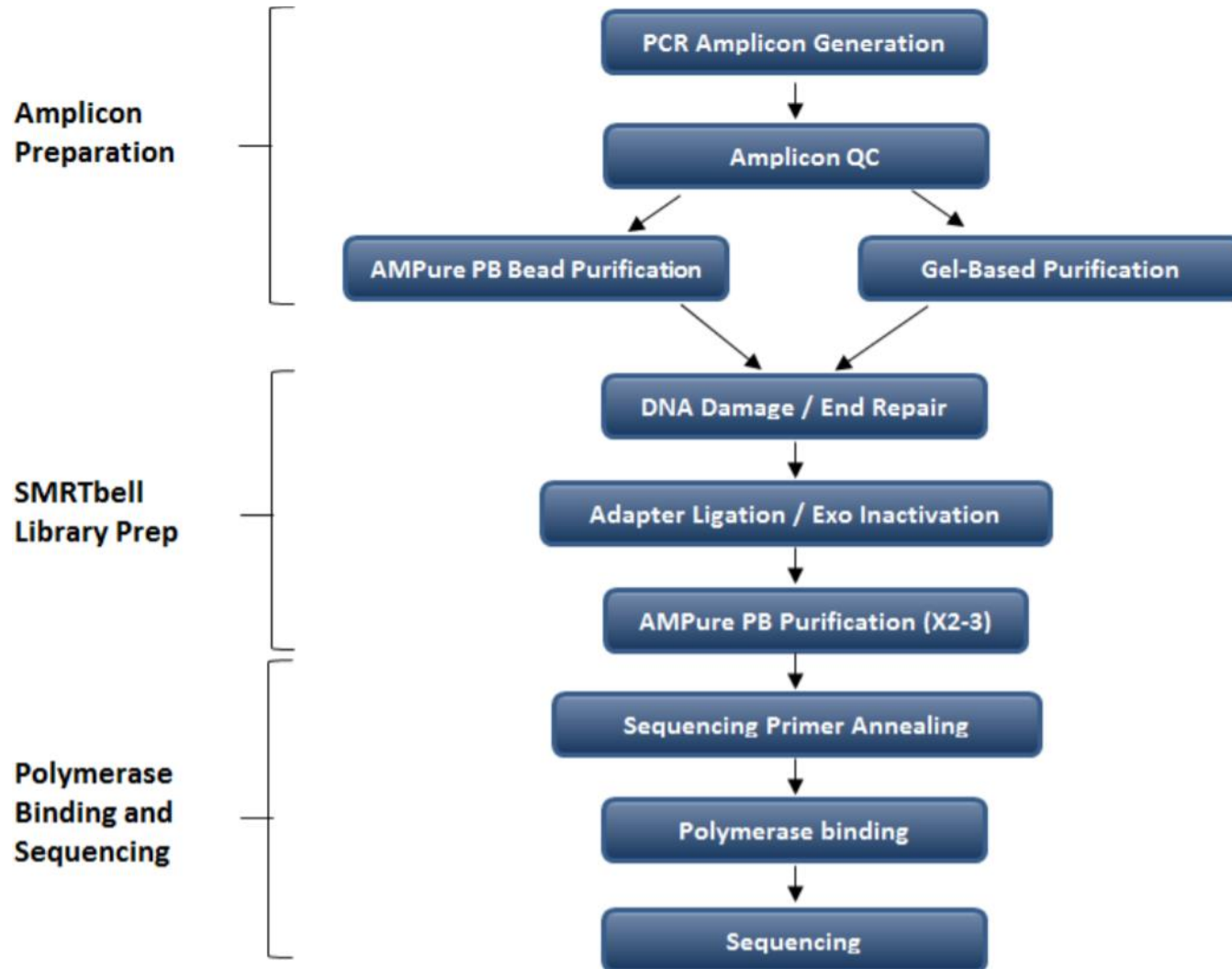
Guidelines for Using PacBio® Barcodes for SMRT® Sequencing

A set of 384 barcodes, each comprised of 16 bp, are custom-designed for the PacBio System. By adding these barcodes to PCR primers, users can perform parallel or multiplex sequencing using SMRT Analysis v1.4 or later. This set of barcodes is ordered for optimal discrimination with SMRT Sequencing; the barcodes at the beginning of the list have maximum sequence differences.

- Follow the recommended input DNA amounts for different amplicon sizes and scale up the reactions accordingly if using more than the default starting input amounts to ensure that the adaptor to template ratio is optimal to reduce concatemer formation

RECOMMENDED SEQUENCING CONDITIONS FOR AMPLICONS (PACBIO RS II)

Insert Size Range	100 bp - 300 bp	301 bp - 999 bp	1 kb - 5 kb	5 kb - 10 kb
Run Protocol	Standard (Diffusion)	Standard (Diffusion)	MagBead OCPW or MagBead Standard	MagBead OCPW or MagBead Standard
Stage Start	No	No	1 kb - 3 kb (No) 3 kb - 5 kb (Yes)	Yes
On-Plate Loading Concentration (nM)	0.1 - 0.2 (custom)	0.2 - 0.45 (custom)	0.010 - 0.025 (custom)	0.025 - 0.040 (custom)
Primer:Template Ratio	5 (custom)	5 (custom)	20 (standard)	20 (standard)
Polymerase:Template Ratio	2 (standard) or 3 (custom)	2 (standard) or 3 (custom)	10 (standard)	10 (standard)

Movie Time	30	45	60	90	120	180	240	360
Bases/Run[1]	3750	5625	7500	11250	15000	22500	30000	45000
Insert Size	Minimum number of passes for movie-limited reads[2]							
100	38	56	75	113	150	225	300	450
300	13	19	25	38	50	75	100	150
1000	4	6	8	11	15	23	30	45
5000	0.8	1.1	1.5	2.3	3.0	4.5	6	9
10000	0.4	0.6	0.8	1.1	1.5	2.3	3.0	4.5

[1] Theoretical minimum read length for a movie-limited read

[2] Based on 125 bases/min, or 2.08 bases/sec, to include slow or paused polymerases

AMPLICON DATA ANALYSIS WITH SMRT ANALYSIS OR PACBIO DEVNET

SAMPLE COLLECTION → **NUCLEIC ACID EXTRACTION** → **AMPLIFICATION** → **QC AMPLICON** → **SMRTBELL CONSTRUCTION/ SEQUENCING** → **DATA ANALYSIS**

Visit [PacBio DevNet](#) to find open-source community data analysis software, documentation, tutorials and PacBio System data sets

- **Resequencing**
  - Map sequencing reads against a reference sequence to identify variants.
  - Example: Targeted SNP detection and validation
- **Long Amplicon Analysis (LAA2)**
  - Generation of reference-free de novo phased consensus haplotype sequences from pooled amplicons
  - Example: Phase full-length HLA allele variation without imputation
- **Minor Variant Analysis (Juliet) (*NEW!*)**
  - Calls minor variants in a heterogeneous data set against a user-provided reference sequence
  - Example: Detection of somatic cancer variants down to 1%
- **Cluster Consensus Analysis (CluCon)**
  - Reference-free deconvolution of distinct genomic species in a complex mixture and determination of relative abundances
  - Example: Analysis of mixtures of near-full length HIV genomes (9 kb)
- **rDNATools**
  - Reference-free deconvolution of distinct genomic species in a complex mixture and determination of relative abundances
  - Example: Analysis of full length 16S sequences to characterize microbial communities

WHERE TO FIND SMRT RESOURCES

http://www.pacb.com/smrt-science/smrt-resources/
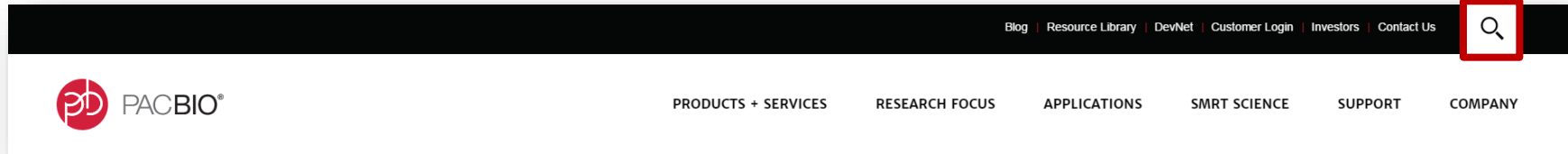
Explore our collection of resources and learn how scientists use SMRT Sequencing to advance their research.

Scientific publications

Explore our database of scientific publications featuring PacBio data.

Conference proceedings

Access conference posters and presentations our customers, collaborators, and internal scientists have presented at various scientific meetings.

PacBio literature

View case studies, brochures, application notes, and more.

Video gallery

Watch our collection of videos, webinars, customer testimonials, and more.

Blog

Read our blog featuring new research, publications, conference summaries, and SMRT Sequencing updates.

Product documentation and training

Visit user documentation for our entire documentation library and training for user training materials.

Q&A and Open Discussion

Q&A AND OPEN DISCUSSION

Frequently Asked Questions

How long can I store my polymerase-bound sample?

- **PacBio RS II:**
  - PacBio recommends that polymerase-bound samples be stored at 4°C and used within 3 days.

- **Sequel System:**
  - PacBio recommends that polymerase-bound samples be stored at 4°C and used within 7 days.

How do I dissociate my polymerase-bound sample from MagBeads?

- Dissociating polymerase-bound sample from MagBeads may damage the sample and is not recommended. PacBio recommends binding sample to MagBeads immediately before sequencing and proceeding with sequencing as soon as possible. If a delay between MagBead binding and sequencing is unavoidable, Customers can store the sample in the dark at 4°C, but delaying sequencing will be at the Customer's own risk. If a MagBead sample has already been aliquoted into a sample plate, the sample plate should be sealed upon storage at 4°C. For Sequel samples, the sample plate should be heat-sealed with the Sequel Sample Plate Foil (P/N 100-667-400). For PacBio RS II samples, the sample plate should be temporarily sealed with an adhesive microplate sealing film and then the sealing film should be replaced with the PacBio RS II Sample Plate Septum (P/N 000-882-901) before sequencing.

How long can I store my MagBead bound sample?

- PacBio recommends that MagBead samples be stored at 4°C in the dark and sequenced as soon as possible.

My MagBeads were accidentally left at room temperature for several hours. Can they still be used?

- In most cases, MagBeads should still be useable by first chilling them at 4°C before use.

My MagBeads / AMPure beads were accidentally stored at -20℃. Is it still okay to use the beads?

- PacBio does not recommend using AMPure PB beads or MagBeads that have been accidentally stored at -20°C because the beads may become damaged and may leach after being frozen. However, Customers *may* use them at their own risk after bringing the MagBeads to 4°C and AMPure PB beads to room temperature.

When preparing >30 kb SMRTbell libraries, can (AMPure-purified and concentrated) sheared gDNA be stored at 4℃ for longer than 24 hours?

- PacBio generally recommends that AMPure-purified and concentrated sheared gDNA be stored for up to 24 hours at 4°C or at -20°C for longer durations. However, if the gDNA is relatively pure (i.e., free of endonucleases), it should be acceptable to store the sheared gDNA sample for 2-3 days at 4°C.

Conditions for shearing gDNA to a size that can support producing ≥30 kb libraries must be determined and verified empirically for each sample. When preparing ≥30 kb SMRTbell libraries using Megaruptor, what is the recommended target shear size if the desired size selection lower cutoff is, for example, 15-20 kb, 30 kb, or 40 kb?

- When preparing ≥30 kb SMRTbell libraries using Megaruptor, the recommended target shear size depends on the size selection lower cutoff to be employed. The Table below may be considered a useful starting point; but empirical optimization and accurate size quantitation are essential:

Library Insert Size (kb)	Size Selection Lower Cut (kb)	Target gDNA Shear Size (kb)
30	15 - 20	30
30 - 40	15 - 20	50
40 - 50	30	60
50 - 60	40	75

Where can I find the Plate Map and sequences of all the primers in the Barcoded Universal F/R Primers Plate - 96 (P/N 100-466-100) product and Barcoded Adapter Plate - 96 (P/N 100-466-000) product?

- To obtain the sequences of the primers used in the Barcoded Universal F/R Primers Plate - 96 Kit, please contact your local Field Applications Scientist, or submit your inquiry through the PacBio Customer Portal (http://www.pacbioportal.com/) or email techsupport@pacificbiosciences.com.
- The Barcode Plate Map Diagram can be downloaded from PacBio's Documentation webpage (http://www.pacb.com/support/documentation/) here: http://www.pacb.com/wp-content/uploads/2015/09/User-Bulletin-Barcode-Plate-Mapping.pdf

There is a 'Barcoding – PacBio RSII and SMRT Analysis 2.3.0 or older' webpage on GitHub (https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Barcoding). Where can I find the latest guidance on PacBio Barcoding recommendations for multiplexed sample preparation for Sequel System / SMRT Link v4.0 (or later)?

- The most up to date information on PacBio multiplexing applicable to SMRT Link v4.0 (or later) can be found here: https://github.com/PacificBiosciences/SMRT-Link/wiki/SMRT-Analysis-Barcoding-Primer

Can I use Illumina 8-bp barcode index sequences for preparing multiplexed samples for PacBio sequencing?

- No; PacBio does **not** recommend using Illumina 8-bp barcode index sequences for preparing multiplexed samples for PacBio SMRT sequencing applications.

How are the 16-bp PacBio barcodes incorporated into the SMRTbell DNA template?

- PacBio uses two approaches:
  - Adding a barcode to end of the standard SMRTbell adapter. The combined adapter is called a Barcoded Adapter.
  - Adding a barcode to the PCR amplicon. This approach involves a two-step PCR reaction workflow. The internal primers for the first PCR are augmented at the 5' end by universal sequences to the target-specific primers. The external primers contain the 16bp barcode at the 5' end and the universal sequences. This approach is called Barcoded Universal Primers.

What are the supported applications for using PacBio Barcoded Adapters and PacBio Barcoded Universal Primers with multiplexed samples? What are not supported applications?

- Supported applications are sequencing of **one species per sample or loci**. Examples of supported applications include: Confirmation of SNPs, resequencing, most Long Amplicon Analysis (LAA) applications, and Sanger sequencing replacement. An exception is HLA typing, which may have 2 species per loci. Multiplexing of HLA has also been demonstrated with the use of additional custom analyses (see PacBio's AGBT 2015 Poster: http://files.pacb.com/pdf/Poster_MultiplexingHumanHLAGenotyping_DNABarcodeAdapters_HighThroughputResearch.pdf)
- Note: The product specifications for the PacBio Barcoded Adapter Kit and PacBio Barcoded Universal Primer Kit are such that the level of barcode oligo contamination in the 96-plate wells should not exceed 5%. Therefore it is possible, though unlikely, to have 1 other contaminant barcode primer/adapter sequence present at levels up to 5%. PacBio does not recommend using the PacBio Barcoded Adapter Kit and PacBio Barcoded Universal Primer Kit for minor variant detection < 10%.

Does PacBio have any specific DNA polymerase enzyme or Kit recommendations for long-range PCR (LR PCR) for generating long DNA amplicon samples for sequencing?

- While PacBio does not recommend a specific enzyme, a high-fidelity enzyme is generally preferred. For example, PrimeStart GXL from Takara and ThermoFisher Phusion Hot Start II DNA Polymerase have given good results to our internal scientists.

Other Discussion Points

- *Do these protocols/tools serve you well for your amplicon sequencing needs?*

- *What other things would you like us to add to our current solutions for amplicon sequencing?*

- *What are your opinions about the fastest growing applications for amplicon sequencing with PacBio?*

  - E.g., clone validation, viral sequencing, 16S/18S, somatic variation, Immune repertoire…….

www.pacb.com