Microbial Multiplexing

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AGENDA

- Part 1: Existing Microbial Multiplexing Procedure
- Part 2: Microbial Multiplexing Kit (RD)

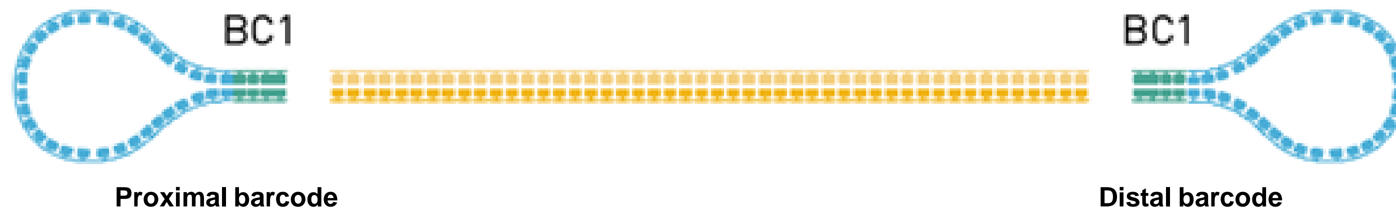
RECENT ADVANCES IMPROVE PERFORMANCE OF MICROBIAL MULTIPLEXING

- Loading bias is minimized
  - Sequel Sequencing Kit 1.2.0 +
  - A non-size selected library may be good enough for most genomes
  - Users can use size selection if desired

- Pre-extension increases detection of the distal barcode

- Improved loading with (ICS v5.0 + SMRT Link v5.0.1+ SMRT Cells new process + Sequencing Kit v2.1) further reduces DNA input requirements into library construction

MULTIPLEXING CHALLENGES

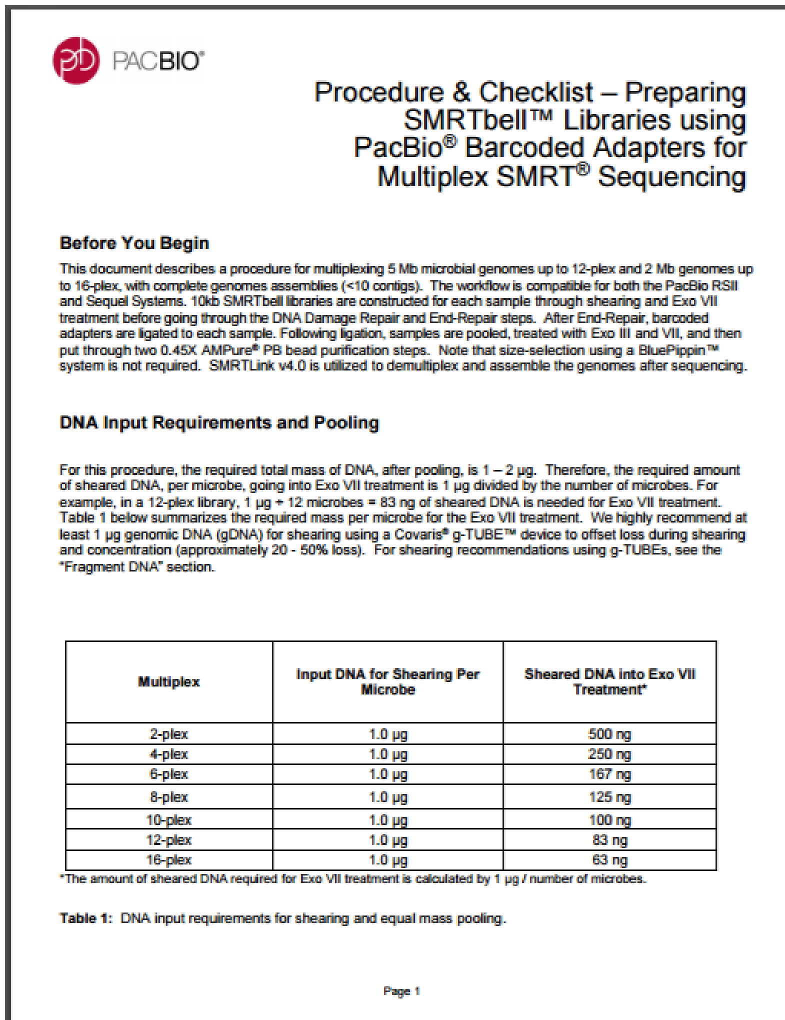


- Proximal barcode is missed
  - Nucleotide incorporation is initiated at the SMRT Cell Prep Station
- Yield of barcoded reads decreases as insert size increases
  - Balance library size = maximize barcoded yield = good assembly (< 10 contigs)
  - Size selection may be necessary (covered by Cheryl Heiner)
- How many microbes can be multiplexed?
  - Depends on project goals

MICROBIAL MULTIPLEXING

- Streamlined library preparation for microbial whole genome sequencing
  - 1 day library prep time
- Reduces project cost for microbial sequencing
  - Library prep
  - Sequencing
- Less than 10 contigs per microbe (depending on project goals)
- Current multiplexing capabilities
  - Sequel System
    - 10-12 ~5 Mb microbes in 1 SMRT Cell
    - 16 ~1.6 Mb microbes in 1 SMRT Cell
  - RSII System
    - 2 ~5 Mb microbes in 1 SMRT Cell

AVAILABLE RESOURCES



PACBIO®

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

Before You Begin

This document describes a procedure for multiplexing 5 Mb microbial genomes up to 12-plex and 2 Mb genomes up to 16-plex, with complete genomes assemblies (<10 contigs). The workflow is compatible for both the PacBio RSII and Sequel Systems. 10kb SMRTbell libraries are constructed for each sample through shearing and Exo VII treatment before going through the DNA Damage Repair and End-Repair steps. After End-Repair, barcoded adapters are ligated to each sample. Following ligation, samples are pooled, treated with Exo III and VII, and then put through two 0.45X AMPure® PB bead purification steps. Note that size-selection using a BluePippin™ system is not required. SMRTLink v4.0 is utilized to demultiplex and assemble the genomes after sequencing.

DNA Input Requirements and Pooling

For this procedure, the required total mass of DNA, after pooling, is 1 – 2 µg. Therefore, the required amount of sheared DNA, per microbe, going into Exo VII treatment is 1 µg divided by the number of microbes. For example, in a 12-plex library, 1 µg ÷ 12 microbes = 83 ng of sheared DNA is needed for Exo VII treatment. Table 1 below summarizes the required mass per microbe for the Exo VII treatment. We highly recommend at least 1 µg genomic DNA (gDNA) for shearing using a Covaris® g-TUBE™ device to offset loss during shearing and concentration (approximately 20 - 50% loss). For shearing recommendations using g-TUBEs, see the "Fragment DNA" section.

Multiplex	Input DNA for Shearing Per Microbe	Sheared DNA into Exo VII Treatment*
2-plex	1.0 µg	500 ng
4-plex	1.0 µg	250 ng
6-plex	1.0 µg	167 ng
8-plex	1.0 µg	125 ng
10-plex	1.0 µg	100 ng
12-plex	1.0 µg	83 ng
16-plex	1.0 µg	63 ng

*The amount of sheared DNA required for Exo VII treatment is calculated by 1 µg / number of microbes.

Table 1: DNA input requirements for shearing and equal mass pooling.

Page 1

Sample Prep

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

Data Analysis:

- http://www.pacb.com/wp-content/uploads/Analysis-Procedure-Multiplexed-Microbial-Assembly-SMRT-Link.pdf

ASM 2017 Poster

- http://www.pacb.com/wp-content/uploads/Lambert-ASM-2017-Multiplexing-Strategies-for-Microbial-Whole-Genome-Sequencing-Using-the-Sequel-System.pdf

PacBio data set

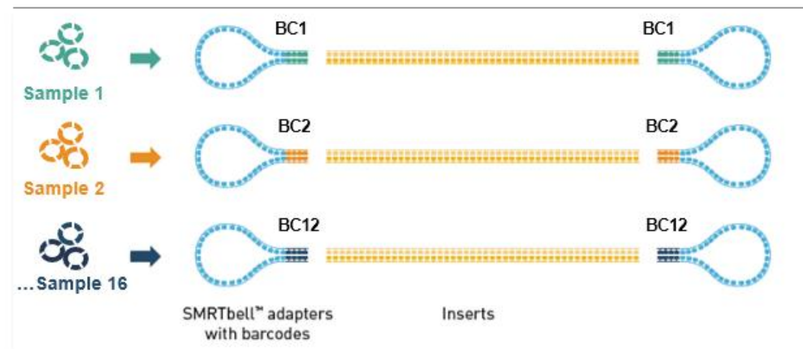
- https://github.com/PacificBiosciences/DevNet/wiki/8-plex-Ecoli-Multiplexed-Microbial-Assembly

MICROBIAL MULTIPLEXING WORKLFOW

- ~10 kb non-size selected library
- Workflow compatible on the PacBio RS II and Sequel System
- Typical library yield based on DNA input after pooling, 20-25%

LIBRARY PREPARATION WORKFLOW

- Each sample is treated with Exo VII to remove 3’ overhangs
- DNA Damage Repair and End Repair
- Barcoded adapters are ligated to each sample
- Pool equimolar
- Subsequent library preparation steps performed in a single tube
- No size selection



DNA INPUT REQUIREMENTS

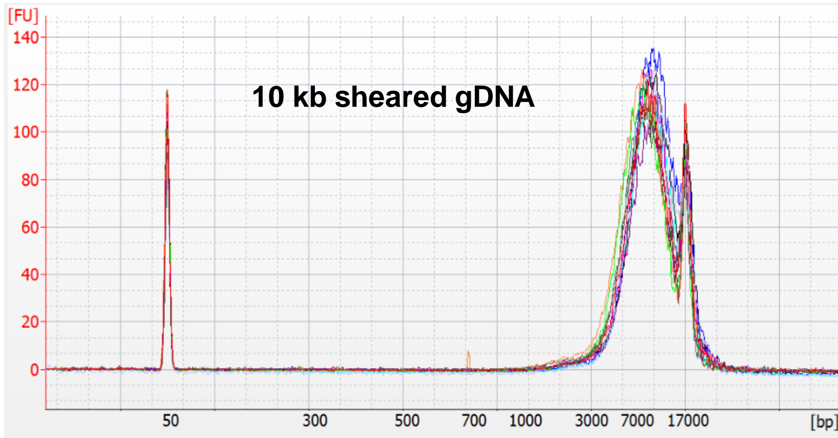
Number of plex	Recommended mass for shearing (ug)	Recommended sheared DNA into Exo VII treatment (ug)
2	1-2	500
4	1-2	250
6	1-2	167
8	1-2	125
10	1-2	100
12	1-2	100
16	1-2	100

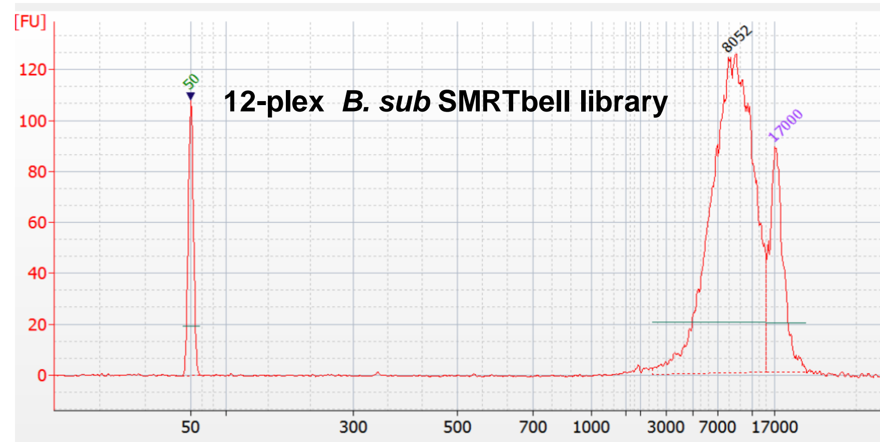
- Shear 1-2 ug DNA per sample
  - More is recommended to account for loss during AMPure
- The amount of sheared DNA required for Exo VII treatment is calculated by 1 – 2 ug ÷ multiplex
- When pooled after ligation, the total mass should be approximately 1 – 2 ug

DNA SHEARING

- To minimize bias, fragment distribution should be similar (+/- 10%)
- Shear genomic DNA to 10 kb using g-TUBEs (or Megaruptor)
  - Recommended shearing conditions using g-TUBE are outlined in the procedure
- Microbes with large repeat regions may not be suitable for multiplexing using a non-size selection approach
  - 20 kb library with BluePippin size selection is necessary
  - Recommend to sequence independently or multiplex less

EXAMPLE OF FRAGMENT DISTRIBUTION

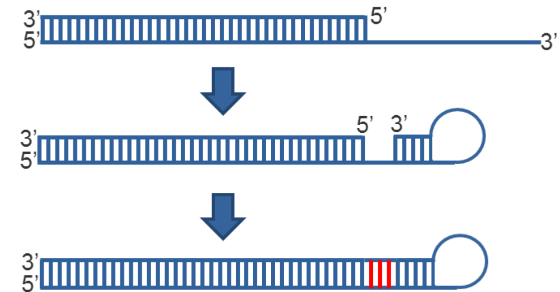




- Highly recommend that samples for one pool should be similar in fragment distribution (+/- 10%)
- Important to perform the following:
  - Use the BioAnalyzer to size the library
  - Use the Qubit system for accurate concentration measurements necessary for pooling

EXO VII TREATMENT IS A CRITICAL STEP

- 3’ overhangs are removed by treating the sheared DNA with Exo VII
- If not removed, single-stranded overhangs can loop back on themselves to form palindromic structures
- % palindromes increases with non-ExoVII treated libraries



Comparison of Exo VII Treated vs. Non-treated Samples

Samples	Total Subreads	Palindromic Subreads	% Palindromes	# Contigs
B. sub (-) Exo VII	205684	23689	11.5	204
B. sub (+) Exo VII	300300	4217	1.4	1
E. coli (-) Exo VII	145000	15866	11.0	101
E. coli (+) Exo VII	347387	7101	2.0	5

AVAILABLE BARCODED ADAPTERS FOR MICROBIAL MULTIPLEXING

Recommended Barcoded Adapters	
BC1054	BC1002
BC1093	BC1070
BC1004	BC1115
BC1080	BC1016
BC1100	BC1101
BC1109	BC1055
BC1032	BC1118
BC1063	BC1048

- Best 16 Barcoded adapters selected from the commercially available kit
- Current solution
  - Barcoded Adapter Plate (available now)
  - Each well is sufficient for two barcoding reactions
- Chosen based on a highest number of subreads and the best ScoreRatio or separation from other barcodes

SEQUENCING RECOMMENDATIONS

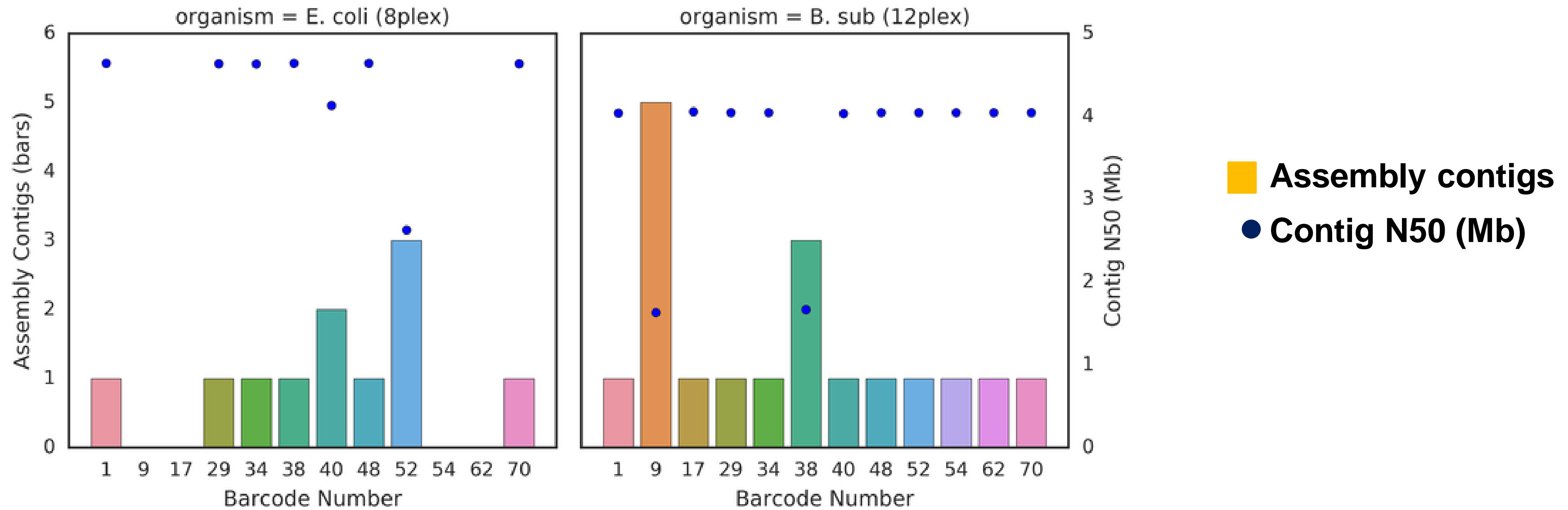
Sequel System

- Recommended On-Plate concentration: 2 pM -8 pM
- Sequel Sequencing Kit v2.1
- 10 hr movies
- Annealing at 0.833 nM; Polymerase Binding at 0.5 nM
- Column clean-up is recommended
- MagBead and diffusion loading

RSII System

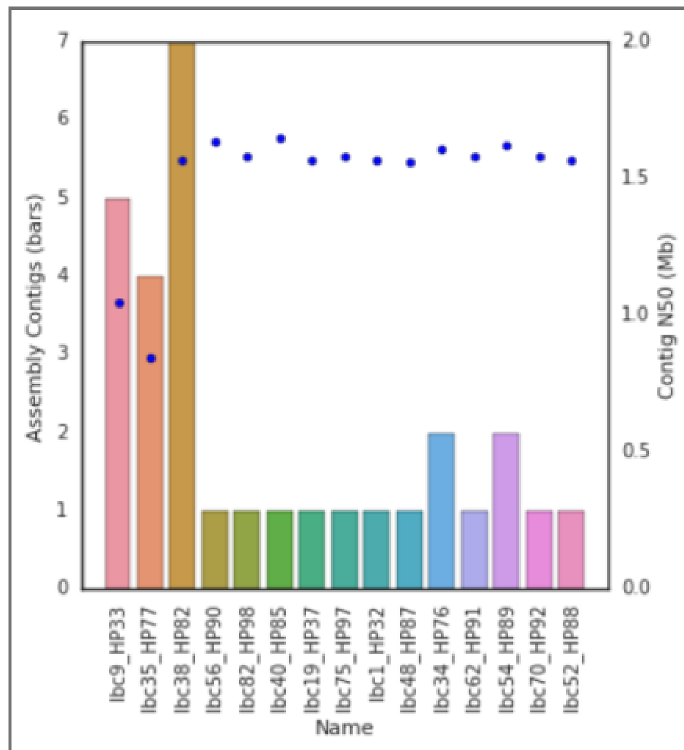
- Recommended On-Plate concentration: 15 pM -30 pM
- P6-C4 chemistry
- 6 hour movies
- Annealing at 0.833 nM; Polymerase Binding at 0.5 nM
- MagBead loading only

8-PLEX *E. COLI* (5 MB) AND 12-PLEX *B. SUBTILIS* (4 MB)



- 1 Sequel SMRT Cell 1M
- Assemblies of *E. coli* (8-plex) and *B. subtilis* (12-plex)
- *E. coli* 8-plex library: 6 of 8 samples with assembly contig N50 ~4.6 Mb
- *B. subtilis* 12-plex library: 10 of 12 samples with assembly contig N50 ~4.2 Mb
- 6-hour movies; v1.2.1 Chemistry (older chemistry)

16 PLEX *H. PYLORI* (1.6 MB)



- Assembly contigs
- Contig N50 (Mb)

- 1 Sequel SMRT Cell 1M
- Small genomes multiplexing
  - 16 strains of *H.pylori**
- 15 of 16 samples assembled with ≤7 Contigs
- 13 of 16 samples with assembly contig N50 ~1.6 Mb
- Lbc17_HP34 showed few contigs possibly due to sample or under-represented
- 6-hr movies, v1.2.1 Chemistry (older chemistry)

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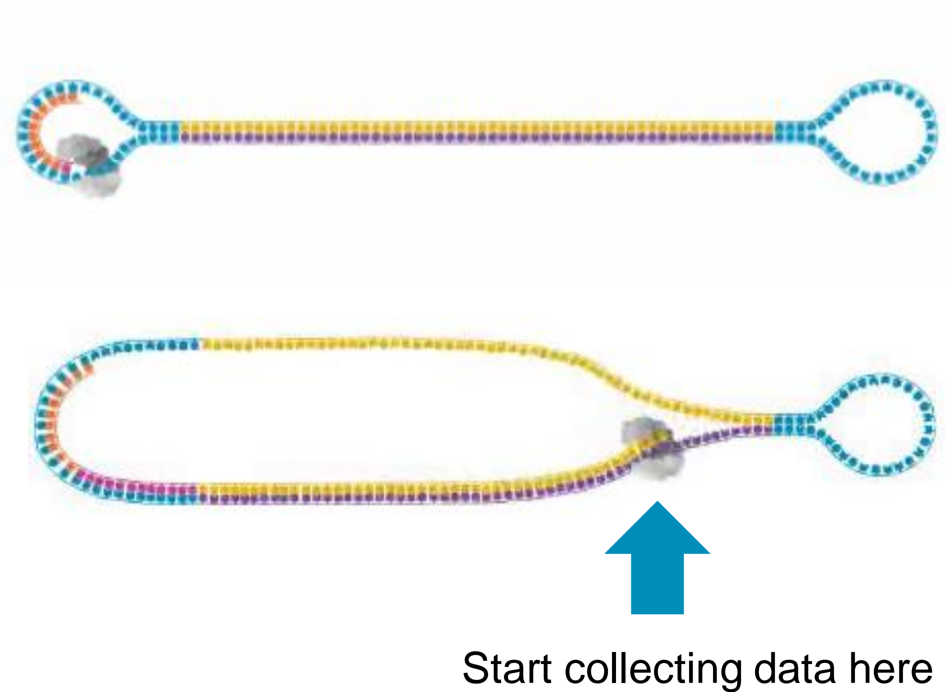
HGAP ASSEMBLY RECOMMENDATIONS FOR PACBIO RS II AND SEQUEL SYSTEM

- 50-fold coverage to achieve good assembly metrics (<10 contigs)
  - Higher coverage is better

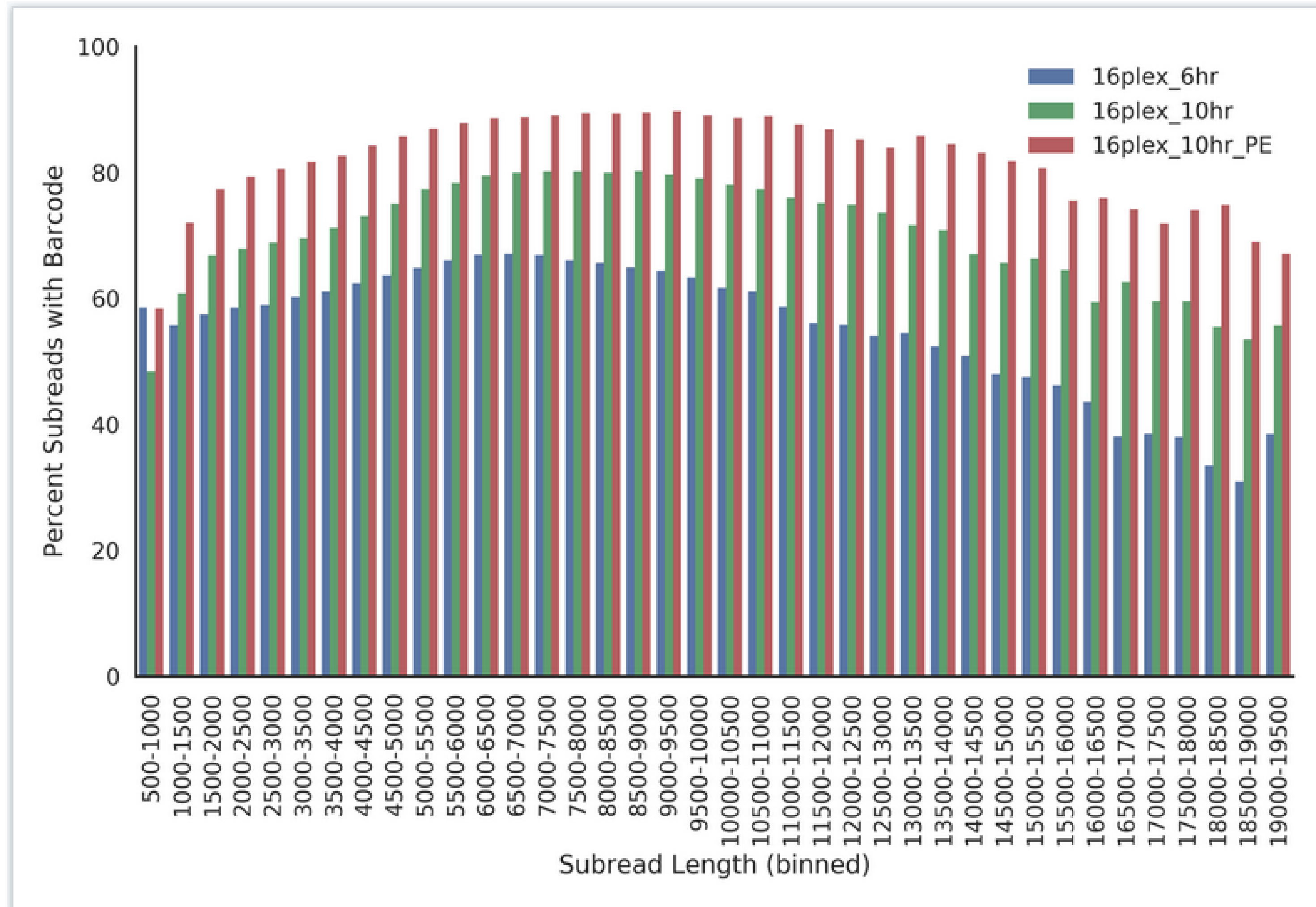
- If higher coverage is required:
  - Sequence additional SMRT Cells
  - Decrease multiplex

PRE-EXTENSION FOR MICROBIAL MULTIPLEXING



- Pre-extension for 120 minutes
- Movie = 10 hours

LONGER MOVIES AND PRE-EXTENSION INCREASE BARCODED YIELD FOR LARGER INSERT SMRTBELLS



SUMMARY

- Workflow compatible both on the PacBio RS II and Sequel System
- Complete assembly with <10 contigs with 50-fold coverage:
  - Multiplex up 16 microbes (<2 Mb)
  - Multiplex up 12 microbes (<5 Mb)
- Recommended # of SMRT Cells:
  - PacBio RS II: 2 microbes = 1 SMRT Cell
  - Sequel System
    - 16 microbes (<2 Mb) / 1 SMRT Cell
    - 12 microbes (4-5 Mb) / 1 SMRT Cell
- 10 hour movies and Pre-extension increase read length and barcoded yield

RESOURCES

Part Number	Product Name
100-514-900	SMRTbell Barcoded Adapter Complete Prep Kit - 96
100-465-800	SMRTbell Barcoded Adapter Kit
100-466-000	Barcoded Adapter Plate - 96
100-465-900	SMRTbell DNA Damage Repair Kit

For more information, see http://www.pacb.com/products/consumables/reagents/ or contact your local sales representative.

References

http://www.pacb.com/wp-content/uploads/2015/09/User-Bulletin-Barcode-Plate-Mapping.pdf
http://www.pacb.com/wp-content/uploads/2015/09/Package-Insert-Barcoded-Adapters-Plate-96.pdf
https://github.com/PacificBiosciences/pbbarcode

www.pacb.com

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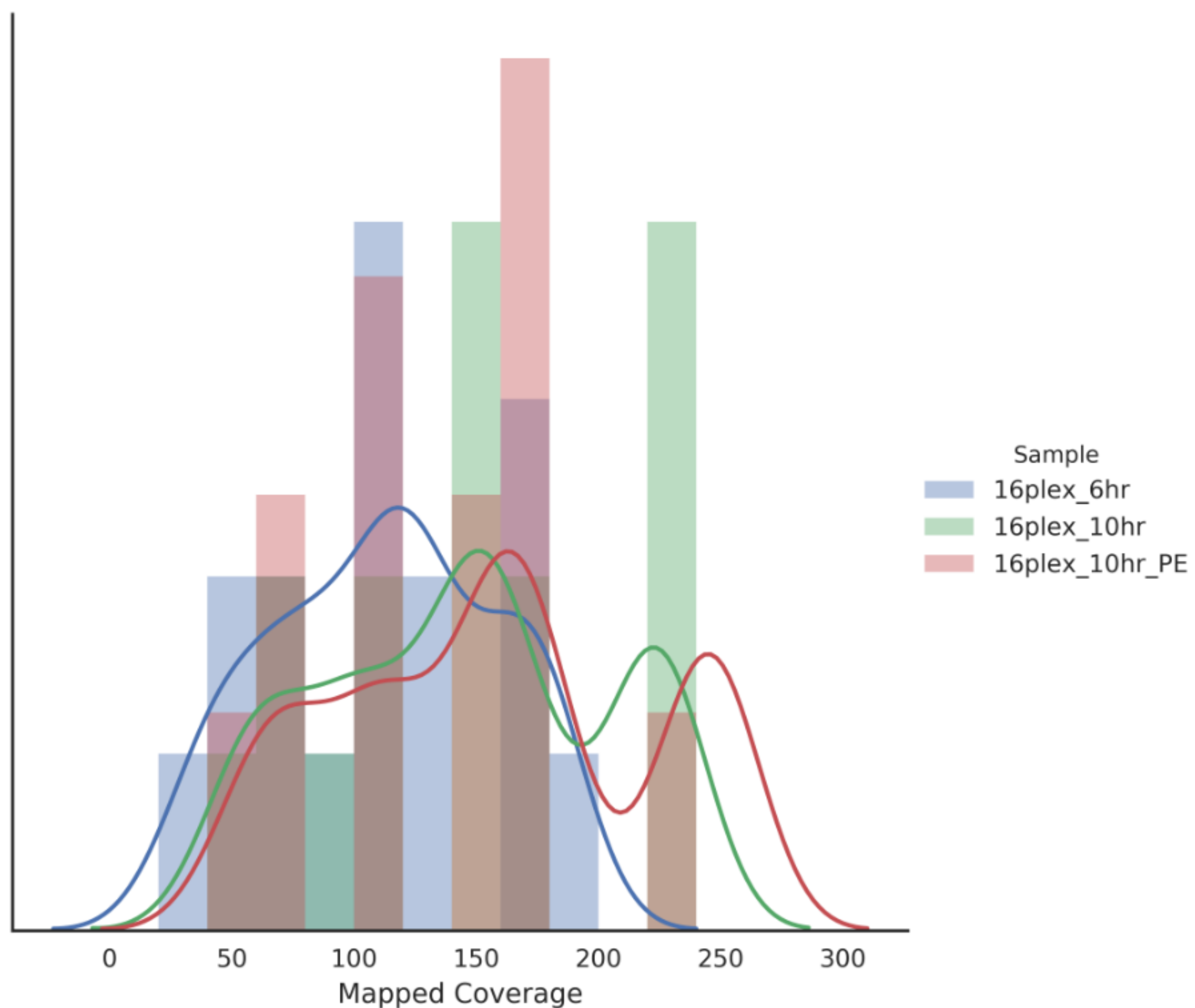
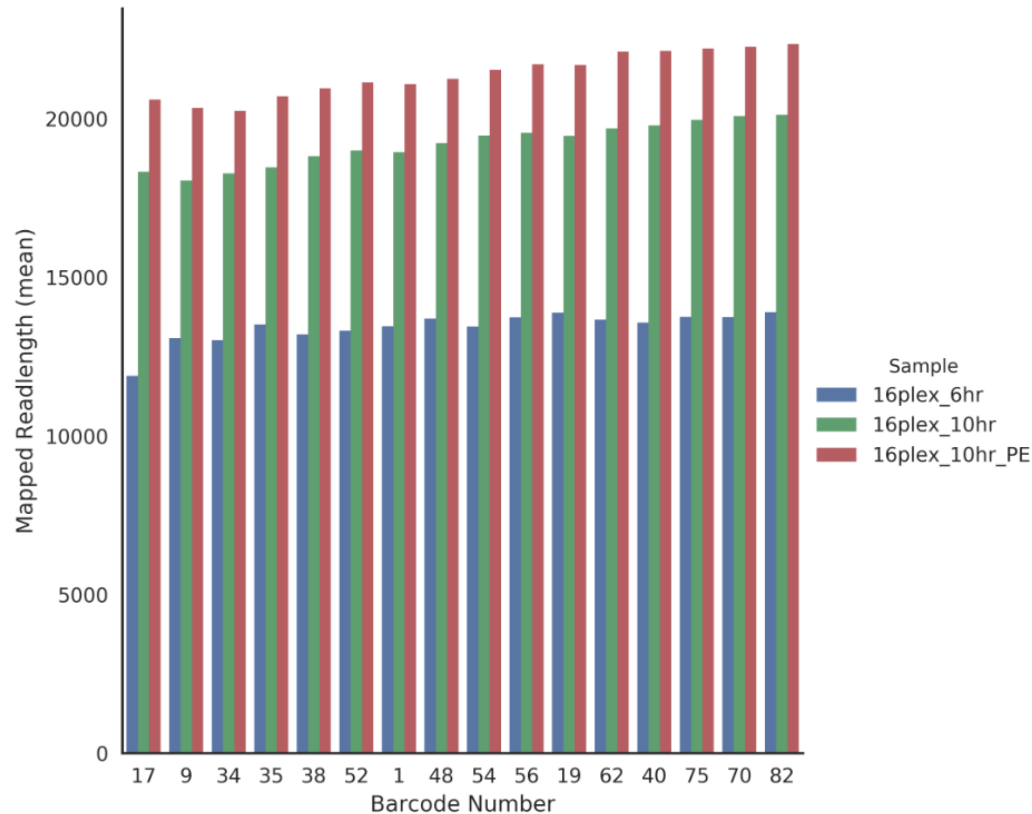
COVERAGE INCREASES WITH 10 HR AND 120 MIN PRE-EXTENSION



IMPROVING MICROBIAL MULTIPLEXING PERFORMANCE



- 10-hour movies increases read length
- Pre-extension provides additional read length increase