Multiplexing Amplicons Up To 10 kb

Increased Study Sizes to Interrogate Genetic Variation

The PacBio® System combines single molecule observation, long-read sequencing, and a low degree of sequence bias to fully characterize genetic complexity. This includes many common variant types with kilobase-size intact fragments. With fully supported end-to-end workflows for multiplexing, and up to ninety-six 10 kb amplicons, study sizes can be increased or large resequencing projects can be performed to better understand this genetic complexity.

- Validated with amplicons up to 10 kb in length
- Formatted to allow multiplexing from 2 to 96 samples
- Structured with workflows for validated or flexible primer designs
- Decreased template preparation cost by potentially 90%
- Decreased time-to-result for template preparation nearly half
- Configured for automated liquid handlers
- Polished workflows from sample to results (using SMRT® Portal Analysis)
- Recommended for single consensus sequence generation per sample

Introduction

PacBio is offering two complete solutions for multiplexing up to 96 amplicons. Both workflows include barcodes to uniquely identify samples, but they differ in how the barcodes are incorporated into the sequencing template. SMRT® Sequencing utilizes a circularized DNA template that is created by ligating SMRTbell™ adapters onto linear DNA fragments. The first multiplexing solution adds the barcode to the SMRTbell adapter, as shown in Figure 1, to yield Barcoded Adapters. Researchers choose the Barcoded Adapter workflow when their PCR primers are already validated (since there are no changes to their existing protocols prior to sequencing on the PacBio System).

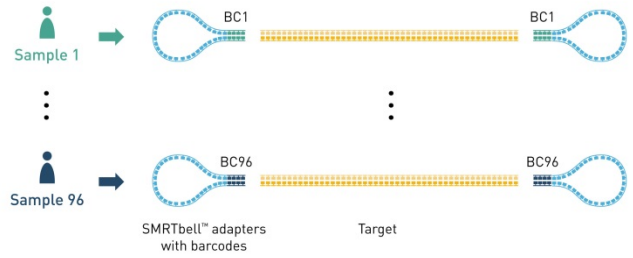

Figure 1. Barcoded adapters are blunt-end ligated to PCR amplicons. By adding the barcode to the standard SMRTbell adapter, researchers can use their existing primer designs to generate amplicons.

When the primer design can be modified, the second multiplexing solution, called the Barcoded Universal Primer workflow, can be utilized. In this method, the barcode is incorporated into the PCR amplicon prior to ligation to the standard SMRTbell adapter. As shown in Figure 2, PCR1 uses internal primers that are a combination of universal and target-specific sequences. Thus, by the end of PCR1, the universal sequences are incorporated into the PCR amplicon. PCR2 is uses primers that hybridize to the Barcoded Universal Primers. These primers are a combination of the universal sequence and the barcode. In this manner, the external primers add the barcode to uniquely identify the sample.

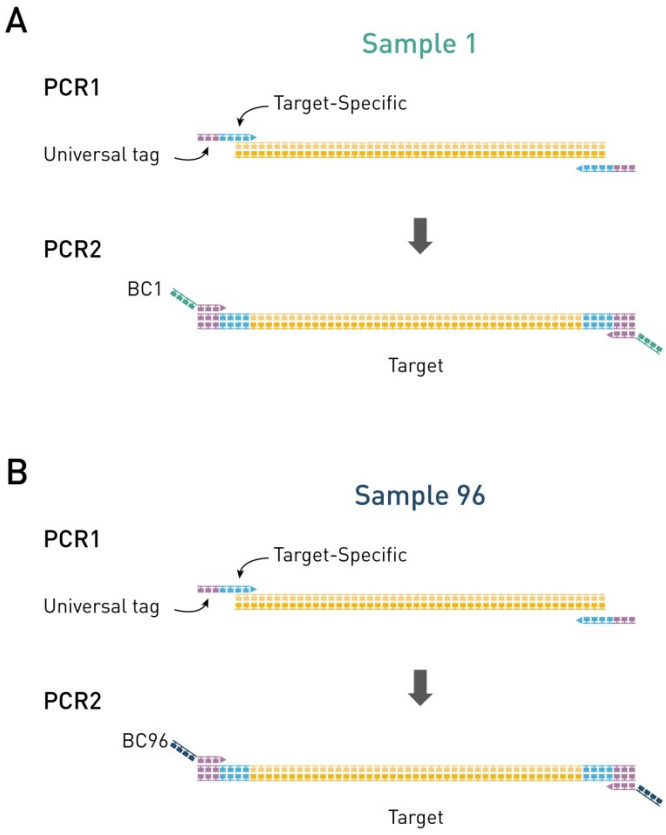

Figure 2. Barcoded Universal Primer workflow involves a two-step PCR process. PCR1 use PCR primers that have universal and target-specific sequences. PCR2 incorporates the barcode. A) Sample 1 incorporating Barcode 1 (BC1); B) Sample 96 incorporating Barcode 96 (BC96).

Complete Barcoding Workflows for Template Preparation to Consensus Sequence

The starting point for each barcoding workflow, as shown in Figure 3, is either gDNA or cDNA. For the Barcoded Adapter workflow[1], the number of steps are reduced because of the combined End Repair and Ligation reactions. The reaction volume is also reduced from 50 µL to 10 µL. However, the End Repair and Ligation reaction must be performed for each sample as the barcode is introduced at this step. The pooling occurs later in the template preparation workflow in contrast to the Barcoded Universal Primer workflow[2].

By adding the barcode into the PCR amplicon in the Barcoded Universal Primer workflow, the pooling of samples occurs prior to the End Repair reaction as shown in Figure 3.

Both barcoding template preparation workflows are completed by annealing the sequencing primer. The following workflow steps are common for either method and involve polymerase binding and sequencing on the PacBio System.

Data analysis is achieved with the standard tools within SMRT Portal Analysis. The analysis includes barcode de-multiplexing and de novo consensus sequence of full-length amplicons.

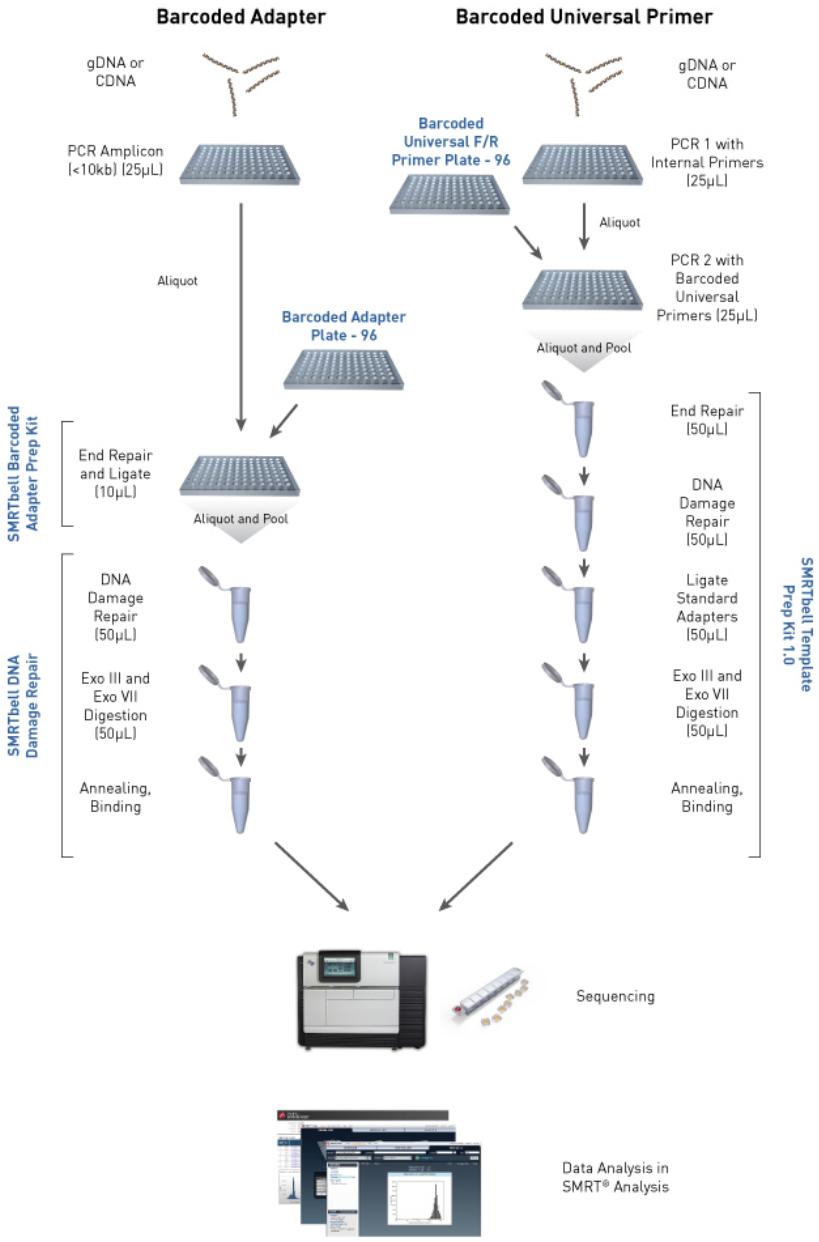

Figure 3. Barcoded Adapter and Barcoded Universal Primer end-to-end workflows. The starting point for both workflows is a DNA template for PCR. Sample pooling is indicated as the workflow changes from a 96-well plate to a single microfuge tube. The PacBio barcoding products required to perform the template preparation are highlighted in blue.

Time and Cost Reduction from Multiplexing Compared to Non-Barcoded Samples

Each barcoding workflow, as described in Figure 3, saves approximately 50% in processing time compared to preparing 96 samples individually using the single-sample, standard SMRTbell Template Prep kit as indicated in Table 1. The time reduction is similar for both barcoding workflows. As previously noted, the Barcoded Adapter workflow requires combined End-Repair/Ligation reactions for each sample. Since these two steps are performed together, the preparation time is approximately equal to the time required for the sequential reaction in the Barcoded Universal Primer workflow.

Template Prep Method	Processing Time for 96 Samples (min)
Standard	465
Barcoded Adapter	230
Barcoded Universal Primers	240

Table 1. Processing time for 96 samples using different template preparation methods. PCR steps are excluded from the processing time.

While the reduction in processing time is similar for both barcoding workflows, the cost reduction relative to the standard template preparation kit can be significantly different for each workflow as detailed in Table 2. At the highest multiplexing of 96-plex, the time to perform the Barcode Adapter workflow is only 10% of the time for the standard template preparation. The savings are generated from the decrease in reaction volume from 50 µL to 10 µL, as shown in Figure 3 (and the pooling of samples from the DNA Damage Repair reaction and subsequent steps). A further 10-fold reduction in cost from the Barcoded Adapter workflow is achieved using the Barcoded Universal workflow at 96-plex for a total cost reduction to ~1% of the standard template preparation. This is accomplished by pooling 96 samples into one standard template preparation reaction.

Decreasing the multiplex from 96- to 8-plex brings the relative cost reduction, when compared to standard template preparation, of the two approaches to a similar level of 16% and 12% for the Barcoded Adapter and Barcoded Universal Primer workflows, respectively. The Barcoded Adapter workflow is relatively insensitive to decreasing the multiplexing while reduction of Barcoded Universal Primer workflow is inversely proportional to the level of multiplexing.

Similar trends comparing the barcoding workflows and the decrease in multiplexing are seen for cost reduction of a complete PacBio System run. While the overall cost savings decreases between the Barcoded Adapter and Barcoded Universal Primer workflow, the difference is smaller for the complete run cost compared to the template preparation cost alone.

A)	Multiplex Level	
Barcoding Method	**96-plex**	**8-plex**
Barcoded Adapter	10%	16%
Barcoded Universal Primers	1%	12%

B)	Multiplex Level	
Barcoding Method	**96-plex**	**8-plex**
Barcoded Adapter	6%	8%
Barcoded Universal Primers	1%	7%

Table 2. Per-sample cost reduction for barcoding workflows compared to the standard template preparation. Two comparisons for either A) the template preparation portion of the workflow, or B) the complete PacBio System run costs. The sensitivity to the level of multiplexing from 96 to 8 samples is examined for each barcoding workflow. All costs exclude PCR costs. The complete PacBio System run assumes a full 8 SMRT Cell run.

Performance

As shown in Figure 4, a series of experiments over 16 SMRT Cells demonstrates the ability to detect the insert correctly.

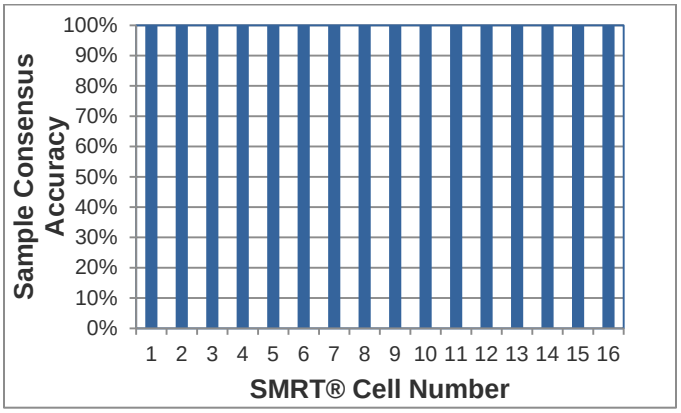

Figure 4. Sixteen runs of a 96-plex of a 1.8 kb insert demonstrate the ability to correctly identify the sample and generate the correct consensus.

In order to generate high quality consensus (e.g., 100% concorcandance), approximately 100X coverage is required, as shown in in Figure 5. Although, for any particular assay, the minimum coverage should be determined experimentally.

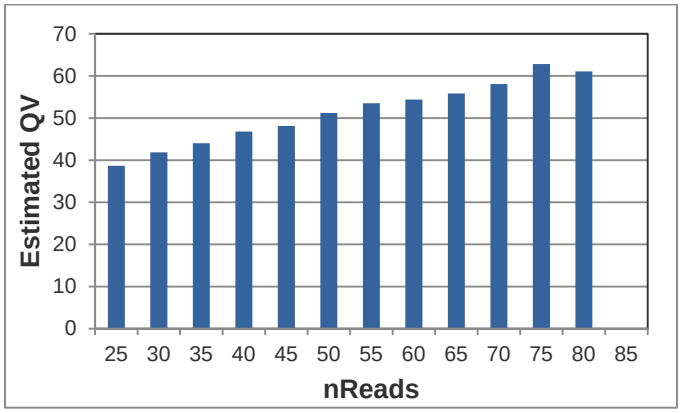

Figure 5. Effect on estimated QV for 5-kb amplicons with increased coverage. The estimated QV plateaus at ~QV60 with 80X coverage. The QV60 is achieved using a 96-plex 5-kb insert dataset. This type of analysis forms the basis of the recommended >100X coverage for each sample.

Demonstration with HLA

A Barcoding Adapter workflow was used for a multi-loci HLA study[3]. For 96 samples, five HLA loci were amplified and each locus was 3-4 kb in length. The five loci were pooled prior to ligating to a single barcoded adapter, per sample, and run over four SMRT Cells. Data analysis was performed through the automated SMRT Portal Long Amplicon Analysis (LAA) pipeline. Of the total 881 expected HLA alleles determined by orthogonal testing, 868 were detected automatically. The remaining thirteen alleles originated from 14 samples and each sample had less than 1500 subreads per sample (on average < 300 subreads / loci). For HLA, a minimum coverage of 500X is recommended because of the assay complexity.

Of the 13 remaining alleles, 9 were recovered by manual data curation and additional sequencing. The remaining alleles were not detected due to less than optimal PCR amplification.

Specifications

The general specifications that are common between the barcoding workflows are described in Table 3.

Description	Specification
Multiplex Level	Up to 96 Samples
96-well plate	Axygen PN 10011-228
Plate Seal	Pierceable Foil
Fill and Reaction Volume (per well)	5 µL and 2.5 µL
Barcode Organization within 96-well Plate	Column Dominant (e.g., A1-lbc1; B1=lbc2; A2=lbc9)
Maximum Amplicon Length	10 kb
Recommended Level of Multiplexing	96 for 2 kb amplicons
	96 for 5 kb amplicons
	8 for 10 kb amplicons

Table 3. List of specifications common to Barcoded Adapter and Barcoded Universal Primer workflows.

Part Number	Product Name
100-466-100	Barcoded Universal F/R Primers Plate - 96
100-514-900	SMRTbell™ Barcoded Adapter Complete Prep Kit - 96
100-465-900	SMRTbell™ Damage Repair Kit
100-259-100	SMRTbell™ Template Prep Kit 1.0

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

References

1) Procedure & Checklist - Preparing SMRTbell™ Libraries using PacBio® Barcoded Universal Primers for Multiplex SMRT® Sequencing
2) Procedure & Checklist - Preparing SMRTbell™ Libraries using PacBio® Barcoded Adapters for Multiplex SMRT® Sequencing
3) Ranade S, et al. (2015) Multiplexing Human HLA Class I & II Genotyping with DNA Barcode Adapters for High Throughput Clinical Research. Advances in Genome Biology & Technology Conference (2015 AGBT) Poster Presentation (Abstract 178). Retrieved April 15, 2015 from https://s3.amazonaws.com/files.pacb.com/pdf/Multiplexing+Human+HLA+Class+I+and+II+Genotyping+with+DNA+Barcode+Adapters+for+High+Throughput+Research%C2%A0.pdf

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