

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.

There are several different options for barcoding samples:

- PCR Primers with Symmetric (Reverse Complement) Barcodes
- PCR Primers with Asymmetric Barcodes
- Barcoded SMRTbell™ Adapters

The decision of which option to use depends on the sample type, insert size, multiplex level, and number of primers desired:

- **Barcoded PCR primers vs. Barcoded Adapters:** in general, it is more efficient to incorporate barcodes into PCR primers, which allows for pooling of samples for SMRTbell library preparations. With barcoded adapters, samples must be prepared separately through the ligation step. Barcoded adapters can be used to multiplex sheared libraries and amplicons that have been already prepared.
- **PCR Primers with Symmetric Barcodes:** in SMRT Analysis, select “**The same on both ends (symmetric)**” from the drop-down menu. Symmetric barcodes allow the most flexibility in insert size and analysis options, as binning requires reading the barcode on only one end of a SMRTbell molecule. This option may be used to multiplex up to 384 samples. Reads with symmetric barcodes have the following structure:



- **PCR Primers with Asymmetric Barcodes:** in SMRT Analysis, select “**Different on each end (paired)**” from the drop-down menu. Asymmetric barcodes may be used to multiplex any number of samples up to 73,536. Fewer barcoded PCR primers are required when multiplexing less than 384 samples with asymmetric barcodes, however, the sequences of both barcodes are required for de-multiplexing. This limits the insert sizes to lengths where barcodes on both ends of a molecule are detected. Reads with asymmetric barcodes have the following structure:



- **Barcoded SMRTbell adapters** are used with sheared libraries and amplicons that are already prepared. A second use is for samples of limited input amounts, where pooling yields SMRTbell libraries of normal concentrations. Note that with barcoded adapters, samples to be multiplexed **must be prepared separately through the ligation step.**

When designing barcode-tailed PCR primers, check for secondary structures in the primers, using a program such as Mfold: <http://mfold.rna.albany.edu/>

Tools for oligonucleotide design are also available on IDT's website: <https://www.idtdna.com/pages/scitools>

For more information on using the barcoding protocol in SMRT Analysis v1.4 and later, go to <https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Barcoding>

A .fasta file with barcode sequences is available on Github: <https://github.com/PacificBiosciences/Bioinformatics-Training/wiki/Barcoding>

Microsoft® Excel® spreadsheets with barcodes for PCR primers, barcoded adapter sequences and simplified ordering of barcoded PCR primers from IDT may be found on the barcodes Shared Protocols page of SampleNet:

<http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0289-to-0384-IDT-Template.xlsx>

<http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0001-to-0096-IDT-Template.xlsx>

<http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0097-to-0192-IDT-Template.xlsx>

<http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0193-to-0288-IDT-Template.xlsx>

<http://www.pacb.com/wp-content/uploads/PacBio-Barcoded-Adapters.xlsx>

More information may be found at the end of this document.

### **PCR Primers with Symmetric Barcodes**

With symmetric PCR primer barcodes, the same barcode (5' to 3') is at both ends of an amplicon, so the reverse PCR primer uses the reverse complement barcode (RC=reverse complement). Barcode 0003 is shown below:

5'-TACTAGAGTAGCACTC-Forward PCR Primer- INSERT-RC Reverse PCR Primer-TACTAGAGTAGCACTC-3'  
 3'-ATGATCTCATCGTGAG-RC Forward PCR Primer- INSERT- Reverse PCR Primer-ATGATCTCATCGTGAG-5'

When ordering barcoded primers, be sure to order the *reverse complement* barcode for the reverse PCR primer. In the example above, the reverse PCR primer would have the following sequence:

5' – GAGTGCTACTCTAGTA - Reverse PCR Primer – 3'

As mentioned above, with symmetric barcodes only one end of a SMRTbell template needs to be sequenced for de-multiplexing a read.

Note: Use of PCR primers with *the same* (instead of reverse complement) barcodes for forward and reverse PCR primers is **not** recommended. Amplicons may form hairpins, leading to lower PCR yield.

### **PCR Primers with Asymmetric Barcodes**

With asymmetric barcodes, completely different barcode sequences are used for the forward and reverse PCR primers for one sample. As mentioned above, asymmetric barcodes may be used if the multiplex level is greater than 384, or to minimize the number of PCR primers required. Note that the sequence of **both** forward and reverse barcodes is needed for de-multiplexing.

Note: When creating asymmetric barcodes, choose barcodes for both forward and reverse PCR primers from the list of **forward** barcodes only.

An example of PCR primers with asymmetric barcodes is shown below:

Forward (using barcode 0001F): 5' – TCAGACGATGCGTCAT - Forward PCR Primer – 3'

Reverse (using barcode 0011F): 5' – CGCGCTCAGCTGATCG - Reverse PCR Primer – 3'

A template containing the above pair of barcodes would have the following structure:

Adapter–Forward Barcode–PCR Primer– INSERT– RC Reverse PCR Primer– RC Reverse Barcode – Adapter

5' – TCAGACGATGCGTCATNNNNNNNNNN–INSERT–NNNNNNNNNNCGATCAGCTGAGCGCG – 3'

### Optional Addition of Pad Sequences to Barcodes

If concerned about unequal ligation efficiencies (due to different sequences at the ends of inserts), consider adding a pad sequence to the 5' end of any of the barcode types. The same pad is added to both forward and reverse PCR primers. To date, PacBio has not observed a need for pads. However, some may prefer to include pads as a cautionary measure against biased representation.

Below is an example of PCR primers with **symmetric** barcodes (0003) and 5-base **padding sequences**:

Forward: 5' – GGTAG-TACTAGAGTAGCACTC - Forward PCR Primer – 3'

Reverse: 5' – GGTAG-GAGTGCTACTCTAGTA - Reverse PCR Primer – 3'

A template containing the above symmetric barcoded PCR primers, **with the padding sequence**, would have the following structure (all sequences 5' to 3'):

Adapter – **Padding Sequence** – **Barcode** – **PCR Primer** – INSERT – **Reverse PCR Primer** – **Barcode** –  
**Reverse Padding Sequence** – Adapter

5'-GGTAG-TACTAGAGTAGCACTC-NNNNNNN–INSERT-NNNNNNN-TACTAGAGTAGCACTC-CTACC-3'

### Barcoded SMRTbell Adapters

Barcoded SMRTbell adapters can be used for multiplexing samples that do not go through PCR amplification, or for samples with limited input. Note that with barcoded SMRTbell adapters, samples to be multiplexed **must be prepared separately through the ligation step**. They can then be pooled for exonuclease treatment and the subsequent steps. For limited input samples, follow all the steps (through ligation) of the relevant Procedure & Checklist for each sample.

There are two options for barcoded SMRTbell adapters, 7-base barcoded adapters and 16-base barcoded adapters. In general, 16-base barcoded adapters will provide more confident barcode identification due to the extra barcode length, and a higher multiplex level is possible. Additionally, 16-base barcodes provide high confidence binning after sequencing through one adapter, whereas 7-base barcodes are suggested for shorter inserts where the barcodes are read multiple times. However, there is a higher initial cost with 16-base barcoded adapters due to the longer oligo length. The table below provides information on each option:

Barcode Length	Maximum Multiplex Level	Oligo Size	Oligo Purification
16 bases	Up to 24*	77 bases	HPLC
7 bases	Up to 12	59 bases	HPLC

\*Additional 16-base barcoded adapters are being tested.

Note: 7-base barcodes and 16-base barcodes were designed separately and are not intended to be combined. This use case has not been optimized or tested.

As an example, the sequence of the 7-base barcoded SMRTbell adapter 1 (adapter sequences shown in lower case) is shown below:

5' –pTATGCTAatctctctctttcctcctcctccgtgtgtgtgagagatTAGCATA – 3'

One strand of a template containing Symmetric sample barcodes would have the following structure:

5' - Adapter -- **Barcode** – INSERT – **Reverse Complement Barcode**– Adapter – 3'

A read with barcoded SMRTbell adapter 1 would have the following sequence:

5' –TAGCATA–INSERT–TATGCTA–3'

## **Annealing Barcoded Adapters to Form Hairpins**

SMRTbell adapter oligonucleotides must be annealed to form hairpins prior to ligation, following the steps below.

1. Prepare 10x annealing buffer:  
100 mM Tris-HCl, pH 7.5  
1 M NaCl
2. Re-suspend or dilute adapters to 20  $\mu$ M in 1X annealing buffer.
3. Anneal as follows (same as annealing primers to SMRTbell templates):  
Incubate at 80°C for 2 minutes, then ramp temperature to 25°C at a rate of 0.1°C/second.
4. Transfer to 4°C location for immediate use or store at -20°C.

No changes are required for SMRTbell library prep, annealing, binding, or loading with these adapters.

## **General Guidelines for Ordering Oligonucleotides**

HPLC-purified oligonucleotides are recommended for SMRT Sequencing applications. A minimum of 85% purity is required, however, the higher the purity, the better.

Excel spreadsheets with barcodes for PCR primers, barcoded adapter sequences and simplified ordering of barcoded PCR primers from IDT may be found on the barcodes Shared Protocols page of SampleNet:

<http://www.pacb.com/wp-content/uploads/PacBio-PCR-Primer-Barcodes-0289-to-0384-IDT-Template.xlsx>

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<http://www.pacb.com/wp-content/uploads/PacBio-Barcoded-Adapters.xlsx>

- Four Excel spreadsheets (one for each 96-wells up to 384-wells) are provided for convenient ordering of barcoded primers from IDT. These Excel sheets can be used to create forward and reverse barcoded primers (see tabs at the bottom each sheet) and then directly uploaded to IDT. At the top of each page, there are instructions on which columns to populate and how to upload to IDT.
- An Excel spreadsheet is also provided for ordering barcoded adapters. Note that there are 2 pages, one with 12-base barcodes and a second with 7-base barcodes.

## **Legacy Barcodes**

The current set of 384 barcodes was not designed to be combined with the legacy set of 96 barcodes. This use case has not been tested nor optimized.