Sequencing Solutions Technical Note

June 2018

How To…

Increase Sequencing Efficiency with the SeqCap EZ Prime Exome

Applications
Whole Exome Sequencing

Products
SeqCap EZ Prime Exome
SeqCap EZ HyperCap

1. Overview

Roche's SeqCap EZ Prime Exome offers comprehensive exome coverage designed for sequencing efficiency. The SeqCap EZ Prime Exome probes tile 98.1% of the coding regions from the CCDS Release 20 (Sept. 8, 2016). It also includes 340 SNPs found in numerous commercial kits to facilitate sample identification throughout sample processing.

The number of sequencing reads required to obtain a certain coverage depth is determined by several aspects of target enrichment performance. The SeqCap EZ Prime Exome has already been optimized to improve sequencing uniformity. Here, we describe an additional modification to the hybridization step in the *SeqCap EZ HyperCap User's Guide, Version 2.1* in order to further increase its efficiency by reducing duplicate rates, enabling higher mean target coverage depths with the same amount of sequencing reads. Using this workflow, we achieved coverage of 96% of the 32.9 Mbp primary target bases at ≥20x depth with 4 GB of sequencing.

Although this method has been effectively used to reduce duplicate rates as presented here, it has not been validated across the complete range of input samples, designs, or library preparation methods. *Please note this alternate method is not supported by Roche.* Variations to the following method may be necessary depending on the variables and requirements of the experiment.

2. METHODS

By increasing the amount of amplified sample library input into hybridization with the *SeqCap EZ HyperCap User's Guide, Version 2.1* workflow, we observed decreased duplicate rates and deeper sequence coverage of targeted regions. Only minor adjustments to the workflow were necessary to implement this change.

Experimental Set-up

Amplified sample libraries were constructed using KAPA HyperPlus Library Preparation Kit with eight different HapMap DNA samples (Coriell Institute) and captured following instructions in the *SeqCap EZ HyperCap User's Guide, Version 2.1* with hybridization preparation using vacuum centrifugation in *Appendix B*. To reduce the effects of variability in library preparation and amplification on capture performance, four replicate libraries per DNA sample were constructed, amplified by LM-PCR (ligation-meditated PCR) and then combined. For each sample, the combined amplified sample libraries were used for all captures, across the range of inputs into hybridization. Eight amplified sample libraries were pre-capture multiplexed per capture, with two replicate captures per hybridization amount. The amount of amplified sample library per sample for each hybridization input amount was tested as follows:

Total combined amplified sample library input into hybridization	Input mass per amplified sample library
1 µg	125 ng
3 µg	375 ng
5 µg	625 ng
10 µg	1.25 µg

Table 1: Amplified sample library per sample for each hybridization input amount

The Multiplex DNA Sample Libraries were combined with 5 µl COT Human DNA and 5 µl HyperCap Universal Blocking Oligos, and then concentrated using the vacuum centrifugation method. The resulting pellets were resuspended in 7.5 µL 2X Hybridization Buffer and 3 µL Hybridization Component A. All other steps were performed according to instructions in the *SeqCap EZ HyperCap User's Guide, Version 2.1*. The amplified captured Multiplex DNA Sample Library pools were sequenced on a HiSeq 2500 instrument (Illumina) with 2x101bp reads using a High Output v4 Kit. Sequencing data were analyzed following the SeqCap EZ data analysis technical note *How To… Evaluate SeqCap EZ Target Enrichment Data, Version 3.0*. Reads were randomly sampled to 40M after adapter trimming and base quality trimming.

Results

Post-capture LM-PCR yields ranged from 1.35 to 4.38 µg and correlated with library inputs into hybridization. The average post-capture LM-PCR yields were 1.50 µg, 2.83 µg, 3.48 µg, and 4.20 µg for 1, 3, 5, and 10 µg inputs into hybridization, respectively.

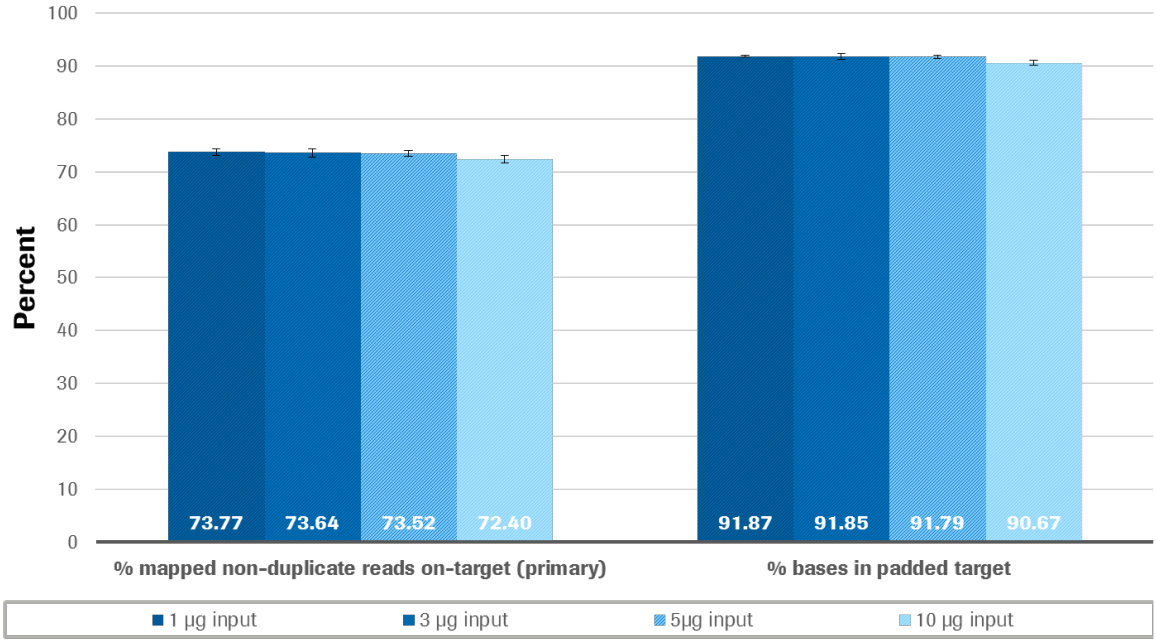

Figure 1: Percent mapped non-duplicate reads on-target (primary target) and % bases in padded target (Percent bases in capture target and adjacent sequenced bases located within 250 bp). Average values for two 8-plex captures (16 samples total) per hybridization input amount are shown with error bars representing standard deviations.

Average percentage of mapped non-duplicate reads on-target (primary) ranged from 72.40 to 73.77% (see *Figure 1*). The percent of mapped non-duplicate reads on-target is the percent of mapped, de-duplicated reads overlapping the primary target region by at least 1 bp, with no padding or buffer. The average percentage of bases in padded capture targets ranged from 90.67 to 91.87% (see *Figure 1*). The percent of bases in padded capture targets is the percent of captured and sequenced bases which are located within 250 bp of the capture target regions of the panel.

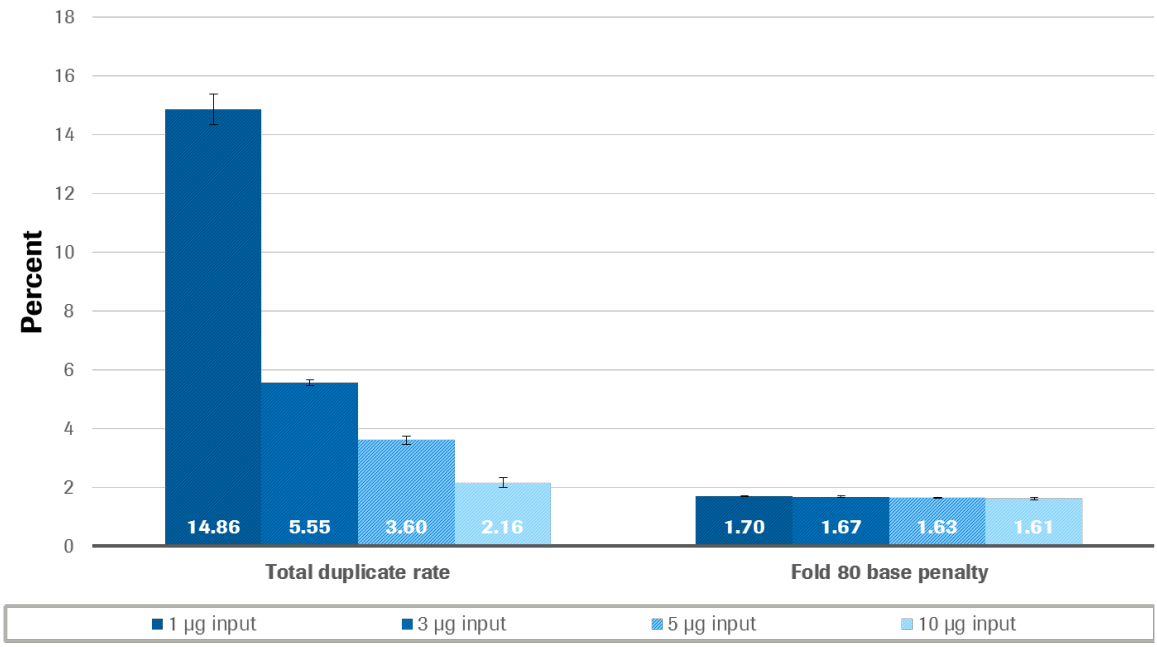

Figure 2: Total duplicate rate and Fold 80 base penalty. Average values for two 8-plex captures (16 samples total) per hybridization input amount are shown with error bars representing standard deviations.

The average total duplicate rate ranged from 2.16 to 14.86% (see *Figure 2*). Total duplicate rate is the percentage of both paired and unpaired aligned reads identified as PCR duplicates. The average Fold 80 base penalties are given in Figure 2 with values from 1.61 to 1.70. The Fold 80 base penalty is a measure of sequencing uniformity and is the fold additional sequencing required to bring 80% of bases to the mean coverage. This is a measure of sequence depth uniformity, where a lower Fold 80 base penalty indicates better uniformity with the best theoretical value equal to 1. Zero coverage regions are excluded for the purpose of calculating Fold 80 base penalty. Percent of bases covered at ≥1X averaged 99.05% for all library input amounts into hybridization and average percentage of bases covered at ≥20X ranged from 93.86 to 96.44% (see *Figure 3*). Mean depth of target coverage on average ranged from 49.09 to 55.04 (see *Figure 3*).

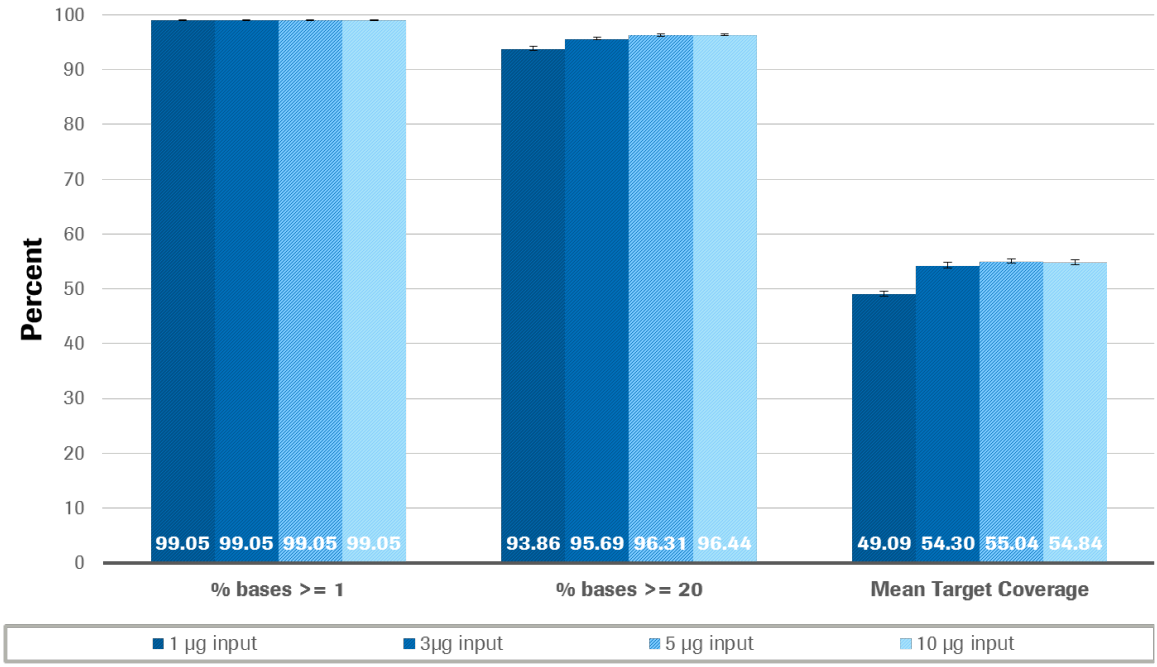

Figure 3: Percent bases covered at ≥ 1X and ≥ 20X and Mean Depth of Target Coverage. Average values for two 8-plex captures (16 samples total) per hybridization input amount are shown with error bars representing standard deviations.

Discussion

The total duplicate rate decreased as library input into hybridization increased (see *Figure 2*). By increasing library input into hybridization from 1 µg to 5 µg, the total duplicate rate dropped by approximately 4 fold to an average of 3.6%. Further, though less dramatic, benefits were observed by increasing the hybridization input amount of the Multiplex DNA Sample Library Pool up to 10 µg. The mean depth of target coverage also improved when the input amount of Multiplex DNA Sample Library Pool into hybridization was greater than 1 µg. Maximum mean depth of coverage was achieved at 5 µg library input and was similar across the 3 to 10 µg library input range (see *Figure 3*). Other metrics such as percent mapped non-duplicate reads on-target, percent bases in padded capture target, uniformity, ≥ 1X coverage and ≥ 20X coverage were comparable across all captures (see *Figures 1*, *2*, and *3*). Detailed guidance on how to execute the workflow described in this document is given in the *Detailed Workflow Instructions for Increasing Input into Hybridization* below.

The SeqCap EZ Prime Exome (170907_HG38_CCDS1X_REZ_HX1) is available on Design Share and can be ordered as SeqCap EZ Share Prime Choice XL (IRN: 4000035460).

Detailed Workflow Instructions for Increasing Input into Hybridization

Note: The results and conclusions described are from experiments that captured 8 amplified sample libraries pooled for each capture reaction (8-plex) using the SeqCap EZ Prime Exome. Higher or lower levels of multiplexing may be possible but were not tested with this protocol and performance may vary. Use of different designs, samples, or library preparation methods may not produce similar results.

1. Follow the *SeqCap EZ HyperCap Workflow User's Guide, Version 2.1* from *Chapter 1* up to and including *Chapter 5: Hybridize the Sample and SeqCap EZ Probe Pool, Step 1*.

2. At *Step 2: Prepare the Multiplex DNA Sample Library Pool*, proceed as follows:

   a. Thaw on ice each of the uniquely indexed amplified DNA sample libraries that will be included in the multiplex capture experiment (generated in *Chapter 4* of the *SeqCap EZ HyperCap Workflow User's Guide, Version 2.1*).

   b. Mix together equal amounts (by mass, see *Experimental Set-up* on page 2) of each of these amplified DNA sample libraries to obtain a single pool with a combined mass of 1-5 µg*. This mixture will subsequently be referred to as the "Multiplex DNA Sample Library Pool".

   * Up to 10 µg total pooled amplified sample library may be used; however, performance improvements were minimal beyond 5 µg in these experiments.

3. Prepare hybridization using Vacuum Centrifugation from *Appendix B* in *SeqCap EZ HyperCap Workflow User's Guide, Version 2.1* with modifications to the amount of Multiplex DNA Sample Library input.

   Note: Bead-based sample concentration has not been tested and is not recommended due to the increased difficulty of eluting hybridization components off of the larger amounts of AMPure XP beads that would be necessary.

   a. Add 5 µL of COT Human DNA (1 mg/mL), contained in the HyperCap Target Enrichment Kit, to a new 1.5 mL tube.

   b. Add the 1 to 5 µg of Multiplex DNA Sample Library from *Step 2b* from the *Detailed Workflow Instructions for Increasing Input into Hybridization* above to the 1.5 mL tube containing 5 µL of COT Human DNA.

   c. Add 5 µL of the HyperCap Universal Blocking Oligos, contained in the HyperCap Target Enrichment Kit, to the Multiplex DNA Sample Library Pool plus COT Human DNA.

   d. Close the lid of the tube and make a hole in the top of the tube's cap with an 18 to 20 gauge or smaller needle.

      Note: The closed lid with a hole in the top of the tube's cap is a precaution to minimize contamination in the DNA vacuum concentrator.

   e. Dry the sample in a DNA vacuum concentrator on high heat (+60 °C).

      Note: Denaturation of the DNA with high heat is not problematic because the hybridization utilizes single-stranded DNA.

   f. To each dried-down sample, add:

      - 7.5 µL of 2X Hybridization Buffer
      - 3 µL of Hybridization Component A

   g. Cover the hole in the tube's cap with a sticker, small piece of laboratory tape, or use a new cap cut from a fresh tube.

   h. Vortex the tube for 10 seconds.

   i. Centrifuge at maximum speed for 10 seconds.

   j. Transfer the spun-down sample to the 4.5 µL aliquot of SeqCap EZ probe pool in a 0.2 mL PCR tube prepared in *Chapter 2* (the entire volume can also be transferred to one well of a 96-well PCR plate).

   k. Mix thoroughly by vortexing for 10 seconds.

4. Centrifuge at maximum speed for 10 seconds. Incubate in a thermocycler using the following program:

   - +95 °C for 5 minutes
   - +47 °C for 16 to 20 hours

   Note: For incubation at 47 °C for 16 to 20 hours, it is important that the thermocycler's heated lid is turned on and set to maintain 10 °C above the hybridization temperature (+57 °C). The sample must remain at 47 °C until it is transferred to the capture beads as described in *SeqCap EZ HyperCap User's Guide Version 2,.1 Chapter 6: Wash and Recover Captured Multiplex DNA Sample, Step 3*.

Continue to *SeqCap EZ HyperCap User's Guide, Version 2.1*, *Chapter 6: Wash and Recover Captured Multiplex DNA Sample*, *Step 3* for washing and recovery. Do not store the sample prior to washing and recovery.

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