Two PCR Strategy for Locus-Specific Deep Sequencing

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1. Extract genomic DNA using Qiagen Blood/Tissue DNeasy kit or other equivalent method.
2. Determine the concentration of each DNA sample using NanoDrop or other equivalent method.
3. Perform PCR #1 reaction(s) using a proofreading enzyme (the following protocol uses Herculase II Fusion DNA Polymerase from Agilent Technologies).
   - Minimize PCR cycles to limit PCR bias. Consider performing multiple different cycle numbers (e.g. 10, 15, 20 cycles) and evaluate on agarose gel.
   - Can try DMSO at increasing concentration 1% to 10%, which often improves specificity. 8% is used in the reaction below.
   - *Amount of genomic DNA (gDNA) for PCR #1 can vary based on experimental needs. On average, a genome from a single cell is approximately 6 picograms. Therefore, 6.6 μg of gDNA represents one million cells. Use adequate gDNA to represent desired number of cells.

   **PCR #1**
   - $X^* \mu$L genomic DNA
   - 10 μL of reaction buffer (5x)
   - 1 μL of 100 mM dNTPs
   - 2.5 μL of 5 μM PCR #1 forward primer
   - 2.5 μL of 5 μM PCR #1 reverse primer
   - 4 μL of DMSO
   - 0.5 μL of Herculase II DNA Polymerase
   - to 50 μL with H$_2$O

   **PCR #1 Cycling Conditions**
   1. 95°C for 2 minutes
   2. 95°C for 20 seconds
   3. 60°C for 20 seconds
   4. 72°C for 30 seconds
   5. Repeat steps 2-4 for minimal number of cycles
   6. 72°C for 5 minutes

   **PCR #1 Primers**
   - **Forward:** TCGTGGGACGGTCAGATGTGTATAAGAGACAG-Locus-Specific-Sequence
   - **Reverse:** GTCTCGTGAGCTCGAGATGTGTATAAGAGACAG-Locus-Specific-Sequence

   *Blue sequence is Illumina Nextera handle sequence*
   *Recommend 20 bp of locus-specific sequence*

4. Perform PCR #2. Each sample will have a unique Illumina Nextera index to allow demultiplexing (see primers below):
   - Minimize cycles to limit PCR bias. Consider performing multiple different cycle numbers (e.g. 10, 15, 20 cycles) and evaluate on agarose gel.
PCR #2
1.0 μL PCR #1 product from step 3 diluted 1:10
2 μL of reaction buffer (5x)
0.1 μL of 100 mM dNTPs
1 μL of 2 μM PCR #2 forward primer
1 μL of 2 μM PCR #2 reverse primer
0.1 μL of Herculase II DNA Polymerase
to 10 μL with H$_2$O

PCR #2 Cycling Conditions
1. 95°C for 2 minutes
2. 95°C for 20 seconds
3. 60°C for 20 seconds
4. 72°C for 30 seconds
5. Repeat steps 2-4 for minimal number of cycles
6. 72°C for 5 minutes

5. Run the PCR #2 product on an agarose gel and gel purify the band of interest.
6. Quantitate DNA by Qubit or other equivalent method.
7. Perform deep sequencing.

PCR #2 Primers

Forward Primers (i5-Index-Handle)
F501 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F502 AATGATACGGCGACCAGCTATACAGTGCTCTATCGTGCCAGCGTC
F503 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F504 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F505 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F506 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F507 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F508 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC
F517 AATGATACGGCGACCAGCTATACAGTAGATCGCTCGTGCCAGCGTC

Reverse Primers (i7-Index-Handle)
R701 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R702 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R703 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R704 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R705 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R706 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R707 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R708 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R709 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R710 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R711 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
R712 CAACGAAAGACGCGATCGCTCGTGCCAGCGTC
Example Locus

Deep sequencing of the -71 DNase hypersensitive site in the HBS1L-MYB interval:

sgRNA (20 bp, chr6:135431630-135431649, hg19)
ACTACTGACATTTATCAACA

PCR #1 primers
Forward: TCGTCGCGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTA
Reverse: GTCTCGTGGGTCTCCAGATGTTATAAGAGACAGCTGCTGGCTTCTTTGCTGTA

Genomic locus (240 bp, chr6:135431513-135431752, hg19)
ACTACTGACATTTATCAACA

PCR #1 amplicon (307 bp amplicon)
TCGTCGCGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTAATCTCGTGGCTTCTTTGCTGTA

PCR #2 amplicon using F501/R701 primers (376 bp amplicon)
AATGATACGGCGACCACCGAGATCTACACTAGATCGCATCGTCGCGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTAATCTCGTGGCTTCTTTGCTGTA

Blue text = Illumina Nextera handle sequence
Red text = 20 bp of locus specific sequence
Bold text = Illumina Nextera index
Green text = Illumina Nextera adapter
Yellow highlight = sgRNA sequence
Blue highlight = PAM sequence
References