**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\* μL 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 H2O

PCR #1 Cycling Conditions

* + 1. 95oC for 2 minutes
    2. 95oC for 20 seconds
    3. 60oC for 20 seconds
    4. 72oC for 30 seconds
    5. Repeat steps 2-4 for minimal number of cycles
    6. 72oC for 5 minutes

PCR #1 Primers

Forward:TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-Locus-Specific-Sequence

Reverse:GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-Locus-Specific-Sequence

*Blue sequence is Illumina Nextera handle sequence*

*Recommend 20 bp of locus-specific sequence*

1. 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 H2O

PCR #2 Cycling Conditions

* + 1. 95oC for 2 minutes
    2. 95oC for 20 seconds
    3. 60oC for 20 seconds
    4. 72oC for 30 seconds
    5. Repeat steps 2-4 for minimal number of cycles
    6. 72oC for 5 minutes

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

**PCR #2 Primers**

Forward Primers (i5-**Index**-Handle)

F501 AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTC

F502 AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC

F503 AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC

F504 AATGATACGGCGACCACCGAGATCTACAC**AGAGTAGA**TCGTCGGCAGCGTC

F505 AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC

F506 AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC

F507 AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC

F508 AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC

F517 AATGATACGGCGACCACCGAGATCTACAC**GCGTAAGA**TCGTCGGCAGCGTC

Reverse Primers (i7-**Index**-Handle)

R701 CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG

R702 CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG

R703 CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG

R704 CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG

R705 CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG

R706 CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG

R707 CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG

R708 CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTCTCGTGGGCTCGG

R709 CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTCTCGTGGGCTCGG

R710 CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG

R711 CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG

R712 CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG

**Example Locus**

Deep sequencing of the -71 DNase hypersensitive site in the *HBS1L-MYB* interval3:

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

ACTACTGACATTTATCAACA

**PCR #1 primers**

Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTA

Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCCTGGGTGACAGAGTGAG

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

CTGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGACTTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTTGATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTCGATTCTACTACTGACATTTATCAACATGGTGGGTGTGATATCTTTTAATCTAATGAGCTATATAACTGCATTTTCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAGTCTCACTCTGTCACCCAGGCT

**PCR #1 amplicon (307 bp amplicon)** TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGACTTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTTGATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTCGATTCTACTACTGACATTTATCAACATGGTGGGTGTGATATCTTTTAATCTAATGAGCTATATAACTGCATTTTCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAGTCTCACTCTGTCACCCAGGCTCTGTCTCTTATACACATCTCCGAGCCCACGAGAC

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

AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGACTTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTTGATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTCGATTCTACTACTGACATTTATCAACATGGTGGGTGTGATATCTTTTAATCTAATGAGCTATATAACTGCATTTTCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAGTCTCACTCTGTCACCCAGGCTCTGTCTCTTATACACATCTCCGAGCCCACGAGAC**TAAGGCGA**ATCTCGTATGCCGTCTTCTGCTTG

*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**

1. Shalem, O. *et al.* Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343,** 84–7 (2014).
2. Canver, M. C. *et al.* BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature* **527,** 192–7 (2015).
3. Canver, M. C. et al. Variant-aware saturating mutagenesis using multiple nucleases identifies regulatory elements underlying trait-associated DNA. Submitted.
4. Illumina Nextera Deep Sequencing: http://support.illumina.com