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 H_2O

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

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_2O

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 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* interval[3]:

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

PCR #1 primers
Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTA
Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCCTGGGTGACAGAGTGAG

Genomic locus (240 bp, chr6:135431513-135431752, hg19)
CTGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGACTTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTT
GATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTCGATTCTACTACTGACATTTATCAACATGGTGGGTG
TGATATCTTTTAATCTAATGAGCTATATAACTGCATTTTCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAG
TCTCACTCTGTCACCCAGGCT

PCR #1 amplicon (307 bp amplicon)
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGAC
TTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTTGATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTC
GATTCTACTACTGACATTTATCAACATGGTGGGTGTGATATCTTTTAATCTAATGAGCTATATAACTGCATTT
TCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAGTCTCACTCTGTCACCCAGGCTCTGTCTCTTATACACAT
CTCCGAGCCCACGAGAC

PCR #2 amplicon using F501/R701 primers (376 bp amplicon)
AATGATACGGCGACCACCGAGATCTACAC**TAGATCGC**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGC
TGCTGGCTTCTTTGCTGTATATCCTGATCACGCTGACTTCCTTCTGCAACTTCTAGATAAGTAAATTTTTTTG
ATTTATCAGGAAGTGTCTTTGGTCTCTCAGTCAATTCGATTCTACTACTGACATTTATCAACATGGTGGGTGT
GATATCTTTTAATCTAATGAGCTATATAACTGCATTTTCTTTTTGTTGTTGTTGTTGTTTTTTGAGATGGAGT
CTCACTCTGTCACCCAGGCTCTGTCTCTTATACACATCTCCGAGCCCACGAGAC**TAAGGCGA**ATCTCGTATGC
CGTCTTCTGCTTG

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