Bulk protospacer-barcode sequencing library preparation

Reagent	1x Reaction
10x Reaction Buffer (Takara)	10 uL
dNTP (Takara)	8 uL
ExTaq polymerase (Takara)	1.5 uL
10 uM FW index primer	5 uL
10 uM RV primer	5 uL
10 ng pDNA	-
NFW	up to 100 uL

Thermocycler Program		
1 cycle	95 °C	1 min
25 cycles	95 °C	30 s
	59 °C	30 s
	72 °C	30 s
1 cycle	72 °C	10 min
Hold	4 °C	∞
Lid temp: 105 °C	Total volume: 100 uL	

1. Note that this PCR protocol indicated above can be performed either on the plasmid directly (recommended input 10 ng), or on gDNA extracted from cells after lentiviral infection (recommended input 2.5-10 ug).
2. After the PCR, run out 1 uL of the product onto a 2% E-gel. You should be able to visualize a clear product at ~350 bp (size will vary slightly depending on choice of forward primer). Formation of additional products is expected and does not interfere with sequencing in our hands.
3. Clean up the reaction with a double-sided 0.6x-1x SPRI cleanup and elute into 40 uL Buffer EB. Quantify libraries for sequencing (we typically run a BioA and take the concentration reported by that, but using the Qubit concentration has also worked well for us in the past).
4. Perform Illumina sequencing of the libraries. In order to sequence through the entire protospacer, a minimum of 60 cycles of sequencing in read 1 is necessary. Also, note that unless you are pooling together libraries prepared with multiple different FW stagger primers, the amplicons are very poor in diversity for sequencing at the beginning of read 1. This means that sequencing will **fail** unless at least 10% PhiX (or some other diverse library) is spiked into the run.

Forward primer sequences and structures

Illumina P5 | Truseq R1 | Sample Index (first 8 bp R1) | FW Priming Site

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F01 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAAGTAGAGTCTTGTGGAAAGGACGAAACACCG-3'
F02 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATACACGATCTCTTGTGGAAAGGACGAAACACCG-3'
F03 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGCGCGGTTCTTGTGGAAAGGACGAAACACCG-3'
F04 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCATGATCGTCTTGTGGAAAGGACGAAACACCG-3'
F05 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATCGTTACCATCTTGTGGAAAGGACGAAACACCG-3'
F06 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCGATTCCTTGGTTCTTGTGGAAAGGACGAAACACCG-3'
F07 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCGATAACGCATTTCTTGTGGAAAGGACGAAACACCG-3'
F08 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCGATACAGGTATTCTTGTGGAAAGGACGAAACACCG-3'
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Stagger Sequences (to improve diversity for sequencing)

Reverse primer sequence and structure

Illumina P7 | i7 Index | Truseq R2 | RV Priming Site

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R01 5'-CAAGCAGAAGACGGCATACGAGATAAGTAGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAGCTCTAGAGGTACCGGATCCC-3'
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** use reverse complement of i7 for demux in sample sheet (CTCTACTT)