For initial plasmid library amplification, we recommend using the protocol described in the GeCKO v2 library documentation as well as the protocols described by Joung et al (2017), *Nature Protocols*.

For preparing paired gRNA libraries for Illumina sequencing:

Set up PCR reaction #1 on ice as follows:

- 25 µL NEBNext High Fidelity 2X Ready Mix
- 2.5 µL of 10 µM forward primer (RKB2713) (0.5 µM final concentration; sequence: TTGTGGAAAGGACGAAACACC
- 2.5 µL of 10 µM reverse primer (RKB2714) (0.5 µM final concentration; sequence: CAAGATCTAGTTACGCCAAGCT
- 1 ng of plasmid DNA
- Water to 50 µL

Run reaction in thermocycler:

| | | |
|---|---|---|
| 98°C | 30 seconds | 1 cycles |
| 98°C | 10 seconds | 20 cycles |
| 65°C | 10 seconds | - |
| 72°C | 1 minute | - |
| 72°C | 2 minutes | 1 cycles |
| 4°C | Hold | |

Purify product using a 0.8X SPRI bead clean-up. Elute in 50 µL of water.

Run the product on a gel. It is important that only a single band is present. If more than one band is present, re-amplify the library using fewer PCR cycles. Over amplifying the library can increase the number of “mis-paired” gRNAs.

Set up PCR reaction #2 on ice as follows:

- 25 µL NEBNext High Fidelity 2X Ready Mix
- 2.5 µL of 10 µM forward primer (RKB2715) (0.5 µM final concentration; sequence: CTAAATGGCTGTGAGAGAGCTCAGTTGTGGAAAGGACGAAACACC)
- 2.5 µL of 10 µM reverse primer (RKB2716) (0.5 µM final concentration; sequence: ACTTTATCAATCTCGCTCCAAACCCAAGATCTAGTTACGCCAAGCT)
- 10 ng of input DNA
- Water to 50 µL

Run reaction in thermocycler:

| | | |
|---|---|---|
| 98°C | 30 seconds | 1 cycles |
| 98°C | 10 seconds | 6 cycles |
| 65°C | 10 seconds | - |
| 72°C | 1 minute | - |
| 72°C | 2 minutes | 1 cycles |
| 4°C | Hold | |

Purify product using a 0.8X SPRI bead clean-up. Elute in 50 µL of water.

Run the product on a gel. It's important that only a single band is present. If more than one band is present, re-amplify the library using fewer PCR cycles. Over amplifying the library can increase the number of "mis-paired" gRNAs.

Set up PCR reaction #3 on ice as follows:

- 25 µL NEBNext High Fidelity 2X Ready Mix
- 2.5 µL of 10 µM forward primer (RKB2717) (0.5 µM final concentration; sequence: AATGATACGGCGACCACCGAGATCTACACACGTAGGCCTAAATGGCTGTGAGAGAGCTCAG)
- 2.5 µL of 10 µM reverse primer (index primer) (0.5 µM final concentration; sequence: CAAGCAGAAGACGGCATACGAGAT***TACAAG***GACCGTCGGCACTTTATCAATCTCGCTCCAAACC)
- 10 ng of input DNA
- Water to 50 µL

For the reverse primer above, the sample-specific barcode is indicated. Different barcodes can be used to accommodate multiplexing.

Run reaction in thermocycler:

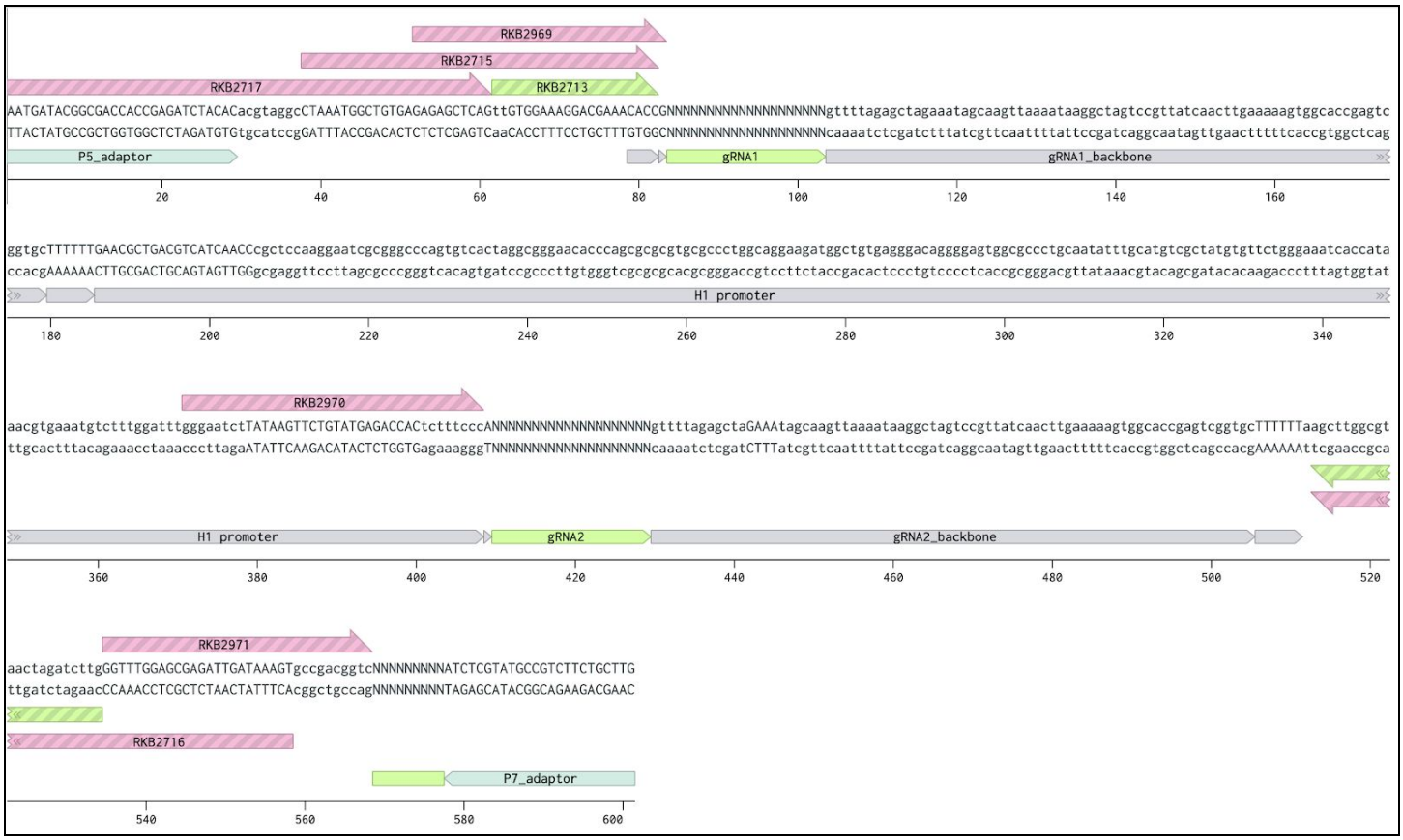
| | | |
|---|---|---|
| 98°C | 30 seconds | 1 cycles |
| 98°C | 10 seconds | 6 cycles |
| 65°C | 10 seconds | - |
| 72°C | 1 minute | - |
| 72°C | 2 minutes | 1 cycles |
| 4°C | Hold | |

Purify product using a 0.8X SPRI bead clean-up. Elute in 50 µL of water.

Run the product on a gel. It's important that only a single band is present. If more than one band is present, re-amplify the library using fewer PCR cycles. Over amplifying the library can increase the number of "mis-paired" gRNAs.

Run product on a gel and/or TapeStation to confirm the library distribution. The final library should be 601 bp.

Here is an example of the final library sequence:




The library can be sequenced on an Illumina sequencer using the following primers:

- RKB2969: GAGAGCTCAGTTGTGGAAAGGACGAAACACCG
- RKB2970: GGGAATCTTATAAGTTCTGTATGAGACCACTCTTTCCC
- RKB2971: GGTTTGGAGCGAGATTGATAAAGTGCCGACGGTC

To capture gRNA1, 20 bp should be sequenced off of RKB2969. The 1st bp of the resulting read is the 1st bp of gRNA1.

To capture gRNA2, 21 bp should be sequenced off of RKB2970. The 1st bp of the resulting read is an A nucleotide (this should be trimmed prior to mapping) followed by 20 bp of gRNA2.

To capture the sample barcode, 6 bp should be sequenced off of RKB2971. The 1st bp of the resulting read is the 1st bp of the barcode.