扩增的sgRNA文库的下一代测序以确定sgRNA分布

用于NGS的文库PCR。

我们提供了NGS引物，可用Illumina接头序列扩增sgRNA靶区。要制备用于NGS的sgRNA文库，请为**10个NGS-Lib-Fwd引物**和**1个NGS-Lib-KO-Rev或NGS-Lib-SAM-Rev条形码引物中的每一个设置反应**，如下所示：

| **Component** | **Amount per reaction (μl)** | **Final concentration** |
| --- | --- | --- |
| NEBNext High Fidelity PCR Master Mix, 2× | 25 | 1× |
| Pooled sgRNA library template from Step 31 | 1 | 0.4 ng μl−1 |
| NGS-Lib-Fwd primer (unique) | 1.25 | 0.25 μM |
| NGS-Lib-KO-Rev (barcode) | 1.25 | 0.25 μM |
| Ultra Pure water | 21.5 |  |
| Total | 50 |  |

使用不同的反向引物和每个文库的唯一条形码，可以在单次NextSeq或HiSeq运行中对不同的文库进行合并和测序。这比在多次MiSeq运行中运行相同数量的文库更高效、更具成本效益。

为了最大程度地减少扩增sgRNA的误差，使用高保真聚合酶（如NEBNext）非常重要。其他高保真聚合酶，如PfuUltra II（Agilent）或Kapa HiFi（Kapa Biosystems），可用作替代品。

使用以下循环条件进行PCR：

| **Cycle number** | **Denature** | **Anneal** | **Extend** |
| --- | --- | --- | --- |
| 1 | 98 °C, 3 min |  |  |
| 2–23 | 98 °C, 10 s | 63 °C, ~~10~~ 30 s | 72 °C, ~~25~~ 30s |
| 24 |  |  | 72 °C, ~~2~~ 10 min |

反应完成后，**将PCR反应物合并**，并根据制造商的说明使用QIAquick PCR Purification Kit纯化PCR产物。

定量纯化的PCR产物，并在2%（wt / vol）琼脂糖凝胶上运行2μg产物。成功的反应应产生∼260至270 bp的敲除文库产物和约270至280 bp的活化文库产物。根据制造商的指示，使用QIAquick Gel Extraction Kit进行凝胶提取。

凝胶提取的样品可在-20°C下储存数月。

根据制造商的说明，使用 Qubit dsDNA HS 检测试剂盒定量凝胶提取的样品。

根据Illumina用户手册，在Illumina MiSeq或NextSeq上对样品进行测序，读取1（正向）80个循环，索引18个循环。我们建议在MiSeq上使用5%PhiX对照或在NextSeq上使用20%PhiX对照进行测序，以提高文库多样性;我们建议文库中每个sgRNA的覆盖率为>100个读长。

**Primer sequences for amplifying sgRNA library and NGS**

| **Primer** | **Sequence (5′–3′)** | **Purpose** |
| --- | --- | --- |
| NGS-Lib-Fwd-1 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTAAGTAGAGGCTTTATATATCT TGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-2 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTATCATGCTTAGCTTTATATATC TTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-3 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTGATGCACATCTGCTTTATATAT CTTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-4 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTCGATTGCTCGACGCTTTATATA TCTTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-5 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTCGATAGCAATTCGCTTTATAT ATCTTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-6 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTATCGATAGTTGCTTGCTTTATA TATCTTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-7 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTGATCGATCCAGTTAGGCTTTAT ATATCTTGTGGAAAGGACGAAACACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-8 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTCGATCGATTTGAGCCTGCTTTA TATATCTTGTGGAAAGGACGAAACAC C | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-9 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTACGATCGATACACGATCGCTTT ATATATCTTGTGGAAAGGACGAAACA CC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-Fwd-10 | AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCC GATCTTACGATCGATGGTCCAGAGCTT TATATATCTTGTGGAAAGGACGAAAC ACC | GeCKO or SAM sgRNA library NGS |
| NGS-Lib-KO-Rev- 1 | CAAGCAGAAGACGGCATACGAGAT**TCGCCTTG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 2 | CAAGCAGAAGACGGCATACGAGAT**ATAGCGTC**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 3 | CAAGCAGAAGACGGCATACGAGAT**GAAGAAGT**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 4 | CAAGCAGAAGACGGCATACGAGAT**ATTCTAGG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 5 | CAAGCAGAAGACGGCATACGAGAT**CGTTACCA**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 6 | CAAGCAGAAGACGGCATACGAGAT**GTCTGATG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 7 | CAAGCAGAAGACGGCATACGAGAT**TTACGCAC**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-KO-Rev- 8 | CAAGCAGAAGACGGCATACGAGAT**TTGAATAG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTCCGACTCGGTGCC ACTTTTTCAA | GeCKO sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-1 | CAAGCAGAAGACGGCATACGAGAT**TCGCCTTG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-2 | CAAGCAGAAGACGGCATACGAGAT**ATAGCGTC**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-3 | CAAGCAGAAGACGGCATACGAGAT**GAAGAAGT**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-4 | CAAGCAGAAGACGGCATACGAGAT**ATTCTAGG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-5 | CAAGCAGAAGACGGCATACGAGAT**CGTTACCA**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-6 | CAAGCAGAAGACGGCATACGAGAT**GTCTGATG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-7 | CAAGCAGAAGACGGCATACGAGAT**TTACGCAC**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |
| NGS-Lib-SAM-Rev-8 | CAAGCAGAAGACGGCATACGAGAT**TTGAATAG**GTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTGCCAAGTTGATAA CGGACTAGCCTT | SAM sgRNA library NGS and barcode (bold) |

以上为张峰Protocol

检测文库完备性时，可以使用一个barcode，多个i5 index [s]，至少四个组合。

Brunello 文库NGS检测说明