**Library amplification protocol**

The library can be amplified by electrotransformation and plasmid preparation. Briefly, about 1 ng of plasmid library in 10 µL should be transformed into 100 µL of XL1-Blue electroporation-competent cells, ideally split across 3 reactions, with the following electroporator settings: 1.75 kV voltage, 200 Ω resistance, and 25 µF capacitance. Recover each electroporation in 1 mL of SOC media, shaking at 37 ºC for 1 hour. Combine and transfer the recoveries to a single 500 mL flask containing 150 mL of LB medium with 100 µg/mL carbenicillin and grow the culture by shaking at 37 ºC for about 12 hours. Purify the plasmid library from the culture by following a standard maxiprep protocol for a low copy number plasmid.

**Experimental protocol for NGS verification**

The library can be verified by NGS with a two-step PCR-based library preparation protocol. If the library will be used for the HT-PAMDA protocol, perform the restriction enzyme digest to linearize the library before proceeding to NGS verification.

PCR1: Amplify the library with the following conditions and thermal cycling protocol:

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Stock concentration** | **Volume (µL)** |
| linearized randomized PAM plasmid library | 5 nM | 1.5 |
| primer pair | 5 µM each | 2.5 |
| Q5 buffer | 5X | 5 |
| dNTPs | 10 mM each | 0.5 |
| Betaine | 5 M | 2.5 |
| MgCl2 | 50 mM | 1.5 |
| Q5 polymerase | 2,000 units/mL | 0.25 |
| Nuclease-free water |  | 11.25 |
| Total |  | 25 |

98 ºC for 2 min, 30X (98 ºC for 10 s, 67 ºC for 10 s, and 72 ºC for 10 s), and 72 ºC for 1 min

Example PCR1 primer pair:

|  |  |  |
| --- | --- | --- |
| oRW1491 | P5 PCR #1 sample barcode primer with CCTG barcode | ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCCCTGGCATGCCTCGTGACCTGC |
| oRW1501 | P7 PCR #1 sample barcode primer with GTCA barcode | CTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGTCACGGTATTTCACACCGCATACGTAC |

\*primers with alternate barcodes can be used for independent samples

Confirm amplification of a 206 bp product by gel or capillary electrophoresis and perform a DNA purification.

PCR2: Append Illumina adapters to the library with the following conditions and thermal cycling protocol:

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Stock concentration** | **Volume (µL)** |
| Template amplicon | 0.125 ng/µL | 2 |
| Primer pair | 5 µM each | 2 |
| Q5 buffer | 5X | 4 |
| dNTPs | 10 mM each | 0.4 |
| Q5 polymerase | 2,000 units/mL | 0.2 |
| Nuclease-free water |  | 11.4 |
| Total (per reaction) |  | 20 |

98 ºC for 2 min, 10X (98 ºC for 10 s, 65 ºC for 30 s, and 72 ºC for 30 s), and 72 ºC for 5 min

Example PCR2 primer pair:

|  |  |  |
| --- | --- | --- |
| OJA1933 | P5 PCR #2 primer Illumina 501 with TATAGCCT barcode (AGGCTATA for NextSeq) (P5-1) | AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT |
| OJA1941 | P7 PCR #2 primer Illumina 701 with ATTACTCG barcode (P7-1) | CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT |

\*primers with alternate barcodes can be used for independent samples

Confirm amplification of a 279 bp product by gel or capillary electrophoresis and perform a DNA purification. Quantify the library and proceed to Illumina NGS.

**Protocol for analysis of NGS verification**

Code and a detailed protocol for analysis of NGS verification can be found on the Kleinstiver Lab’s HT-PAMDA github repository: <https://github.com/kleinstiverlab/HT-PAMDA>

**Representative NGS verification results**

NGS verification reveals the distribution of PAM sequences (the randomized bases) in the library. In a typical library, the representation of each sequence should be roughly normally distributed. For example, the most abundant 4 nt PAM is less than three times that of the least abundant 4 nt PAM (**Fig. 1**).

A screenshot of a cell phone

Description automatically generated

**Fig. 1**. **Anticipated library distribution.** The representation of each of the 256 4nt PAMs in an example substrate library. The abundance of each PAM in the library is represented as frequency distribution histogram (left), or represented as from least to most abundant based on raw read counts (right). The narrow distribution of 4 nt PAMs in the untreated substrate library reflects a balanced library.

**Schematic representation of the libraries.**

A screenshot of text

Description automatically generated

**Fig. 2. Schematics of substrate libraries harboring randomized PAMs.** (**a**,**b**) the plasmid libraries are comprised of a fixed spacer sequence adjacent to a randomized sequence in place of the PAM, where the randomized sequence is on the 3’ or 5’ end of the spacer (**panels a** and **b**, respectively). Libraries with 3’ or 5’ randomized PAMs can be used to characterize the PAM requirements of CRISPR-Cas9 or -Cas12 enzymes, respectively.