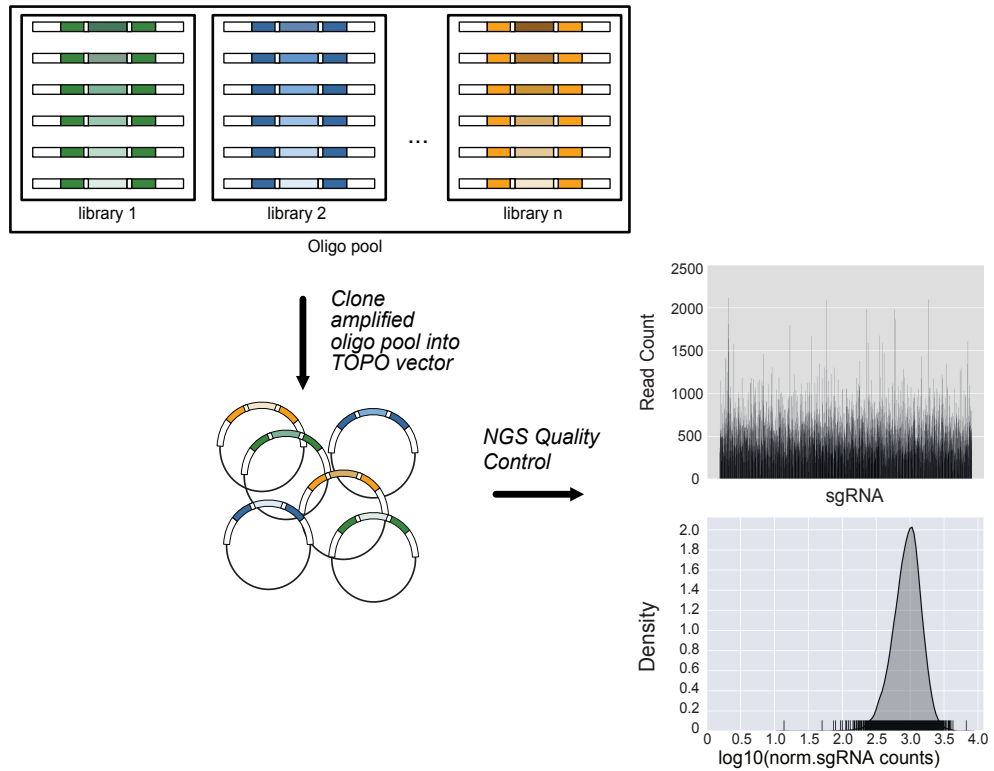


A



B

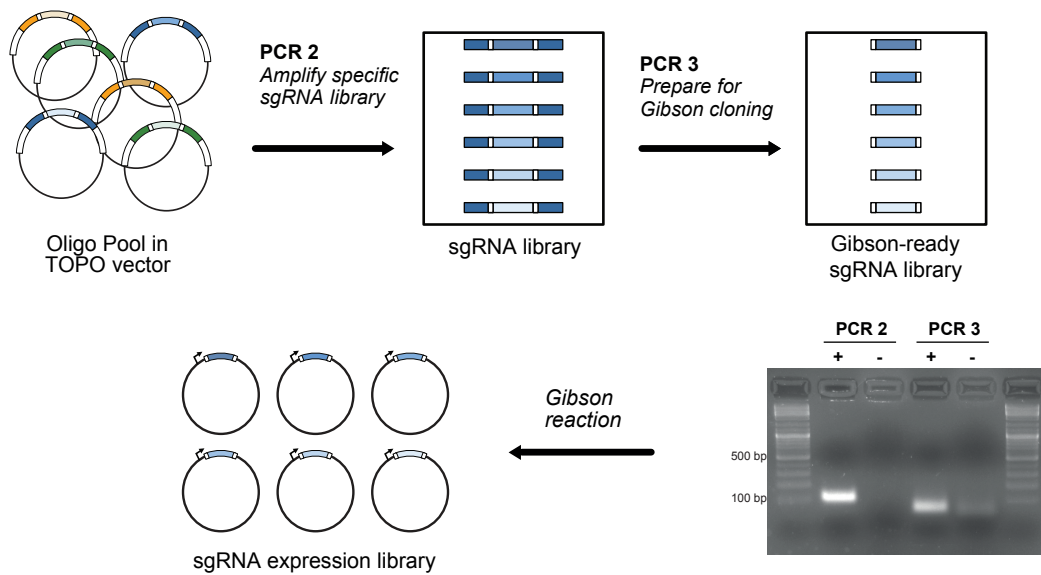


Figure S1: Overview of the CLUE cloning process with quality controls. (A) PCR amplification and cloning of the oligo pool into the TOPO vector is controlled by NGS. The bar graph represents raw read counts for each sgRNA of the entire pool. Normalized read counts and distribution of the sgRNAs are visualized in a density-rug plot, with each rug representing a single sgRNA. The narrowness of the distribution is a quality control measure. **(B)** PCR amplification of a given sgRNA library (PCR 2) and subsequent amplification with primers binding the vector homologous sequences to obtain smaller, Gibson-ready fragments (PCR 3), is controlled on an agarose gel, showing efficient PCR amplification and decreasing fragment size due to the nested PCR design (+ with PCR template, - water control). The Gibson-ready sgRNA library fragments are subsequently cloned into a sgRNA expression vector.

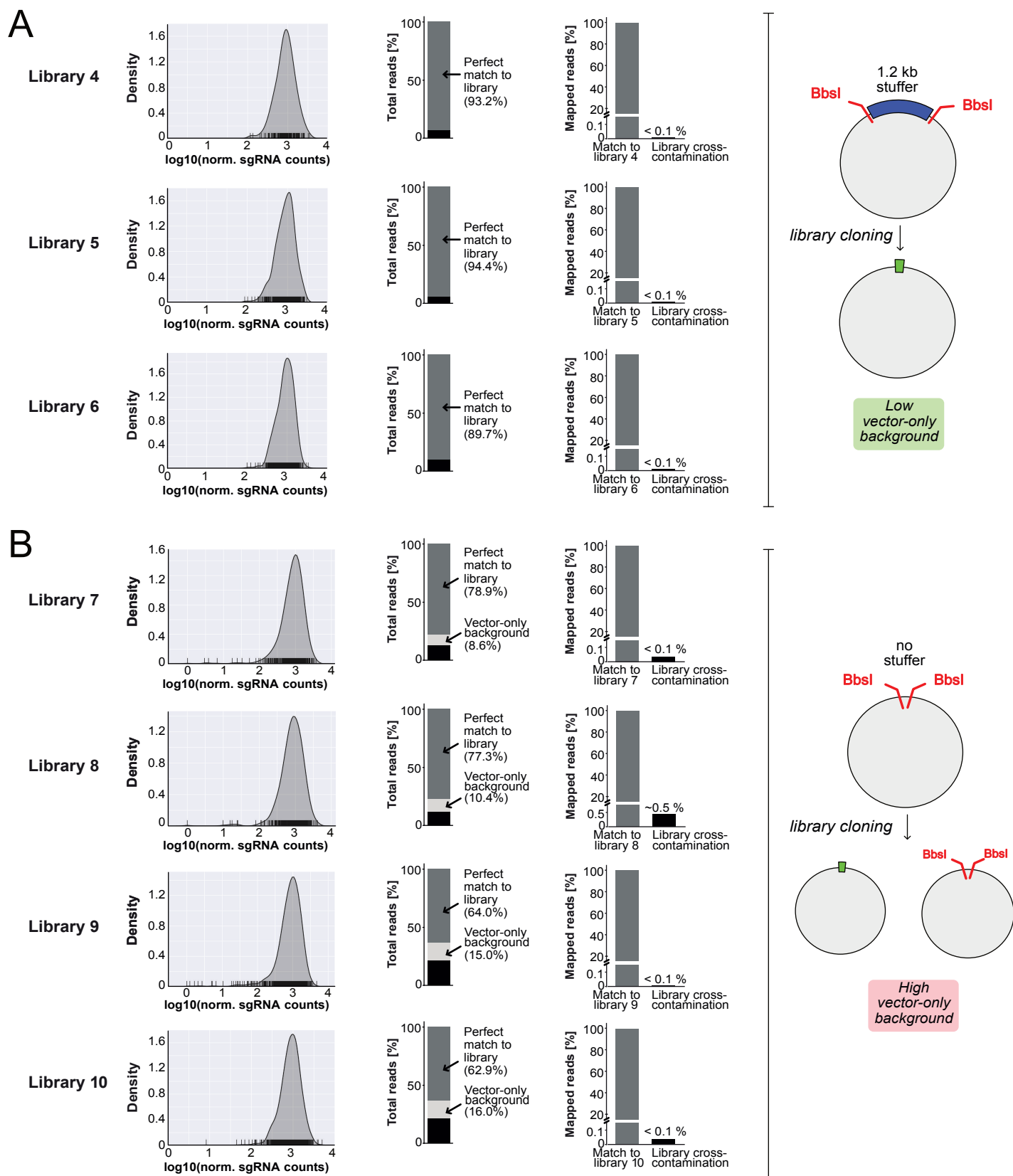


Figure S2: Distribution and quality assessment of seven sgRNA libraries cloned with CLUE. (A) sgRNA libraries cloned into a sgRNA expression vector containing a stuffer sequence for improved vector linearization. *First panels:* Density-rug plots revealing the distribution of all sgRNAs of the respective library. Each rug represents one sgRNA. *Second panels:* Percentage of reads from a NGS run, which could be (dark grey bar) or could not be mapped (black bar) to sgRNA sequences within the oligo pool or to the empty vector (light grey bar), respectively. Only perfect consensus to the sgRNA sequences was counted as successful mapping. *Third panels:* Distribution of mapped reads from a NGS run to the sgRNA library that was amplified in the given experiment (match to library) versus reads mapped to any other sgRNA library present in the original oligo pool (cross-contamination). **(B)** sgRNA libraries cloned into a sgRNA expression vector not containing a stuffer sequence. Panels show data as described in **(A)**.

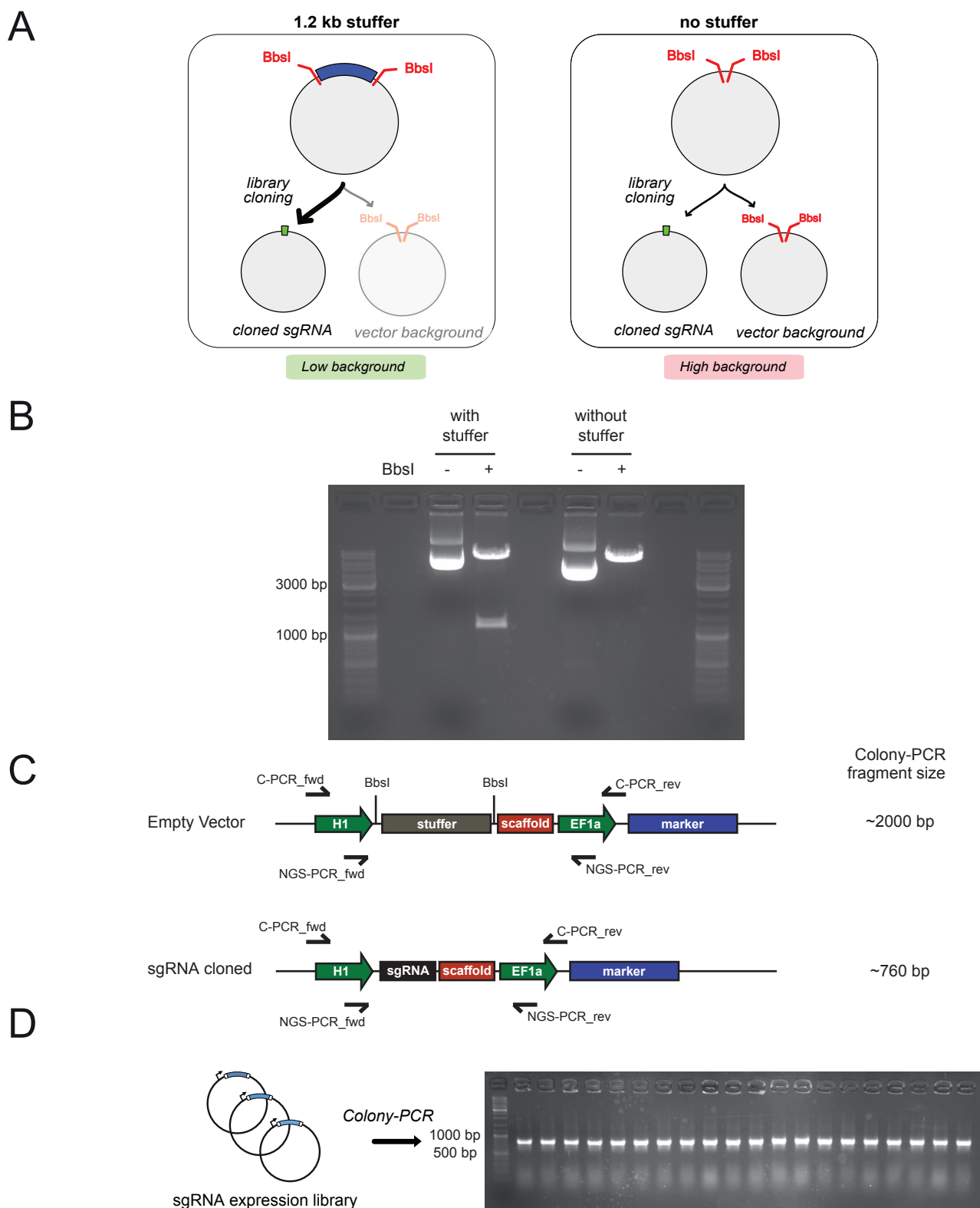


Figure S3: sgRNA library cloning quality assessments. **(A)** Schematic representation of cloning procedures using sgRNA expression vectors with or without stuffer sequences for vector linearization, respectively. Presence of a stuffer improves vector linearization and subsequent sgRNA library quality by limiting empty vector background clones. **(B)** Example agarose gel from vector linearization with BbsI, comparing the presence and absence of a stuffer sequence. **(C)** Schematic representation of sgRNA library cloning into an expression vector with stuffer sequence. Successful cloning leads to a smaller vector due to replacement of the large stuffer with a sgRNA, which can subsequently be analyzed by Colony-PCR. **(D)** Example Colony-PCR of a sgRNA library cloning experiment with an expression vector containing a stuffer sequence. All clones analyzed show a band at 760 bp, confirming successful sgRNA cloning and low empty vector background.

Supplementary Tables

Table S1: Reference sgRNA libraries used by Clue

Type	Species	Reference	Addgene ID
CRISPRko	Human	Wang et al., 2015	#1000000067
CRISPRko	Murine	Doench et al., 2016	#73632
CRISPRi	Human	Horlbeck et al., 2016	#1000000090
CRISPRi	Murine	Horlbeck et al., 2016	#1000000092
CRISPRa	Human	Horlbeck et al., 2016	#1000000091
CRISPRa	Murine	Horlbeck et al., 2016	#1000000093

Table S2: Sequences of DNA adapters for the specific amplification of sgRNA libraries

Forward Adapter	Reverse Adapter	Experimentally determined Tm
ATTTTGCCCCTGTTCTT	CCAGTTCATTTCTTAGGG	59°C
TCACAACTACACCAGAAG	GCAACACTTTGACGAAGA	62°C
CTGTGTAATCTCCGACAC	GCCTTTGCATGTTGTGGA	62°C
GCGTGTGTTGAATTCCACT	AAATTTCTCGTCGGCTC	62°C
CTCAAAGTCGGTGCAACTGT	CGCCAAGATGCTTGGTAAT	62°C
AGGATCTCTAGCCTCAAA	GATGAAGCATCGTAACTG	62°C
AACTGCGATCGCTAATGT	GTTCTCCAGTGCCTTATT	58°C
CTTCTACCGAACATACAG	TCGCGTTATGCTGTATGT	58°C
CGTAGCGTTTTGTACACG	CACGCTCAAAAAGCGTAC	62°C
GGCATGCTGCAATAACCT	AAACCGGTGAGCTGGAAT	62°C
CCGTAACCTATTCTAGCC	GCAGCCCGAATACTTTCA	Not tested
ATACGTAAACCCGTATGG	TCTAGCCTACAATTCACG	58°C
CCCTAAGCATTGCGGAAA	ATTGTTAGCGCCACAATC	Not tested
CATTCTAGCCCTTCGAG	AACTCAACTTGGCAGGAA	Not tested
GGATTGGACAAGCTAGTT	TAATACAAGGCCGCGTTG	Not tested
GGGGGAAAAGGATCGATT	TCACGTGTAAACGATGCG	Not tested
ACCCAGTTGTGAATATC	TCGGATGACCCCTAGAAAG	Not tested
TATGAACCACTAAGGCGT	CGTAAAGTCTGCTGGTGA	Not tested
GCTGGTGTTATGGTGAA	AAGTCGTTTTTGGACCGC	Not tested
GAGCACACACAAGAATGA	TAGCGACAACGTCACAAC	Not tested
CACACTATGTCATCCGCA	CCTTTAGCAAGCAAAGCC	Not tested
TAGTCAGAGAGTCGAGAG	GGTTTGGAGCCATTAGTT	Not tested
GTAGACAAATGCTTGGAC	TCCCAGTCTAGTATGAGG	Not tested
AAGGCCCAAGTCGCTTTT	CCGCCTTTTCAAGTGAT	Not tested
TCGATCCGGGAGTATACA	CCTACAAGAGTTCGACAC	Not tested
ACATCCTGGTTACTTGGC	TGCTCTCGATCATAGCCT	62°C
GAAATATTAAGGGCGGCT	TTCGGTCAATAGAGTCGG	Not tested
AATATGTCCCCTTCCTAC	CACTCTGTTCGGAAAAC	Not tested
TTGGAAAGCAGTACTGCA	GATGTGTAAGTGGGCCTA	Not tested
AGAGATCCGGTGTCAAAG	CCCAGTTCAATCGCTCAA	Not tested
CGACTGGACGGATTTTGA	CGTTCCTCCGCCTTATTA	Not tested
TGCGTAATGCATGTGATC	TGCTATTAAGATCCTCCC	Not tested
ATCATCAATCACTCCCCG	TCGATCTTGAGAAGCAGT	Not tested
GTCTATTGAAGTACCTGC	TATCTTGGAGGAGCATCG	Not tested
TGGCATTGCTTCGTCAAG	CATGACGACTTCACCCAT	Not tested
CCAACATGACGTTCTGTC	GAACGTCGAGTAAATGTC	Not tested
TTTACGGTCCACCATTTG	AACGTGAGGATTAGCGCT	Not tested
GACGTGGACTTGGACAAA	GAAGTGTGCGATTTGCAG	Not tested
TGTTTGACAATCTCGGGC	GCACATAAGTACCACTCC	Not tested
CAAGCGCTAAGCACGAAA	AGTGATATCTACCGCGTG	Not tested
TTATTAGTTTGTGCCCGC	AAAAGCATACAGGCACCA	Not tested

CTCGGGATAGTTATACTC	CTTAGTGTAGATTTGGGC	Not tested
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Table S3: List of Primers used

Primer Name	Sequence (5' → 3')	Purpose
Pool_ampl_f	TGCGGATCATTCAATACGG	Initial oligo pool amplification (PCR1)
Pool_ampl_r	CGCCATAACGATGTTTGAG	Initial oligo pool amplification (PCR1)
H1_f	CTGTATGAGACCACTCTTTCCC	PCR for cloning-ready fragments (PCR3)
Scaff_r	TGTTTCCAGCATAGCTCTTAAAC	PCR for cloning-ready fragments (PCR3)
M13_f	GTAAAACGACGGCCAG	Control PCR of TOPO pool
M13_r	CAGGAAACAGCTATGAC	Control PCR of TOPO pool
P7-TOPO-5p	CAAGCAGAAGACGGCATACGAGATNNNNNNNN GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGAGCGGATAACAATTTACACAGG	NGS rev primer for TOPO pool
P7-TOPO-3p	CAAGCAGAAGACGGCATACGAGATNNNNNNNN GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGGTTTTCCCAGTCACGACGTTG	NGS rev primer for TOPO pool
P5-H1_f	AATGATACGGCGACCAACGAGATCTACACNNN NNNNNTCGTCGGCAGCGTCAGATGTGTATAAG AGACAG[N] _n GTATGAGACCACTCTTTCCCG	NGS fwd primer for TOPO pool and cloned library
P7-EF1a_r	CAAGCAGAAGACGGCATACGAGATNNNNNNNN GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGAAAAAGGCGGAGCCAGTACA	NGS rev primer for cloned library
C-PCR_f	CGATCTGCAATATTTGCATGTCGC	Colony PCR
C-PCR_r	TCGCTAGCTCTAGAGTAGGCGC	Colony PCR

*[N] NGS barcodes, [N] nucleotides for staggers