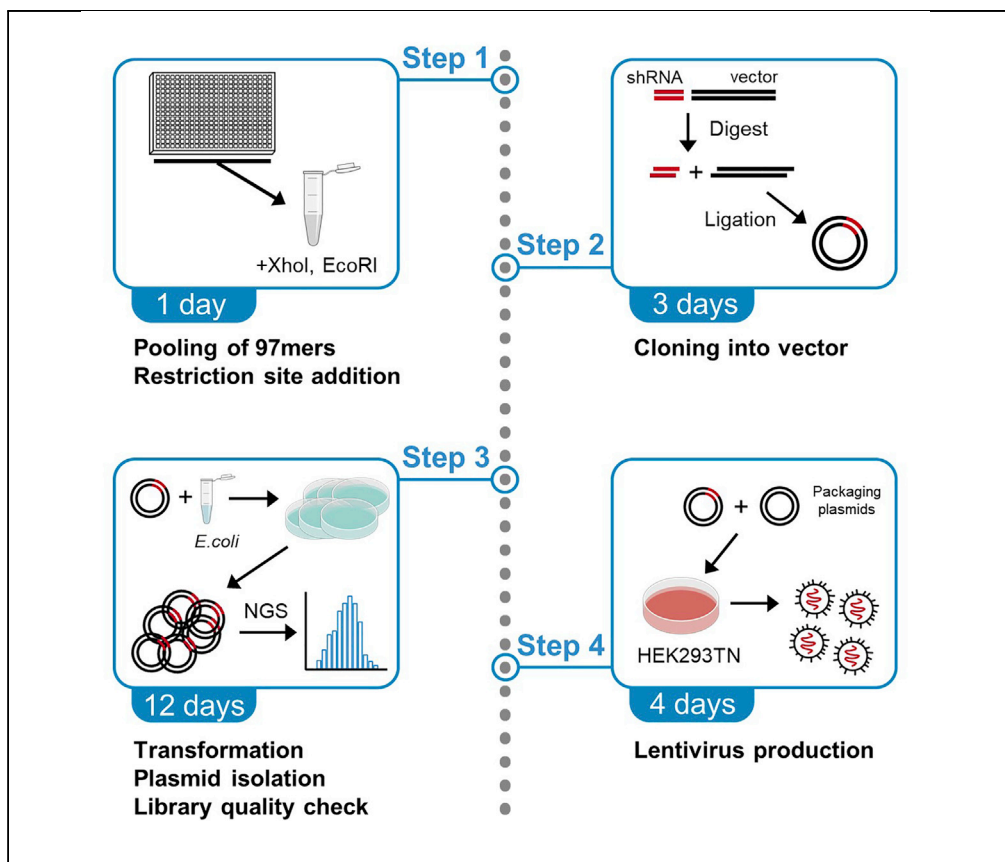


Protocol

Generation of a pooled shRNA library for functional genomics screens



Here, we detail a protocol for the generation of pooled short hairpin RNA (shRNA) libraries. We cover the design of optimized miR-E backbone shRNAs, cloning into a Tet-on vector system, and transformation of competent bacteria. We also describe library quality check by next-generation sequencing, and finally the production of lentiviruses. This protocol will generate high-quality inducible libraries suitable for both genome-wide and targeted functional genomics screens, allowing the high-throughput interrogation of protein depletion effects in the cell system of choice.

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Highlights

A comprehensive protocol for use in functional genomics screens

Detailed steps from shRNA design up to lentivirus production

Compatible with a wide range of commercial vectors and bacterial strains

High-quality libraries will ensure the reproducibility of subsequent screens

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Protocol

Generation of a pooled shRNA library for functional genomics screens

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SUMMARY

Here, we detail a protocol for the generation of pooled short hairpin RNA (shRNA) libraries. We cover the design of optimized miR-E backbone shRNAs, cloning into a Tet-on vector system, and transformation of competent bacteria. We also describe library quality check by next-generation sequencing, and finally the production of lentiviruses. This protocol will generate high-quality inducible libraries suitable for both genome-wide and targeted functional genomics screens, allowing the high-throughput interrogation of protein depletion effects in the cell system of choice.

For complete details on the use and execution of this protocol, please refer to Papadopoulos et al. (2022).

BEFORE YOU BEGIN

The protocol below describes the generation of a library encompassing around 600 shRNAs cloned into a Tet-on doxycycline-inducible vector. An earlier version of this protocol was used for a similar targeted screen using the same vector (Jung et al., 2017).

Choice of vector backbone

⌚ Timing: 1 day

The choice of vector is critical, as inducible shRNA activation optimizes selection during cell culture, particularly for drop-out screens. We used the lentiviral doxycycline-inducible pLT3GGmirEPPIR vector, a derivative of the pRRL vector (Fellmann et al., 2013). However, this protocol is compatible with commercially available vectors, such as pGIPZ (Open Biosystems), pTRIPZ (Thermo Fisher Scientific), and pINDUCER11 (Addgene: 44363) (Meerbrey et al., 2011).

1. Carefully examine the sequence of the vector (Figure 1).
 - a. Check whether the promoter is constitutively active or under the control of a Tet system. In our case, the PGK is constitutively active for the puromycin resistance and rTA3 (reverse tetracycline-controlled transactivator). The rTA3 binds to the tet-operator after doxycycline addition and induces the expression of the shRNA and GFP (Figure 1).

Note: rTA3 is not shown in Figure 1, but follows immediately after the IRES depicted. While two tet-operator sequences are shown, a total of seven sequences are present in the vector.



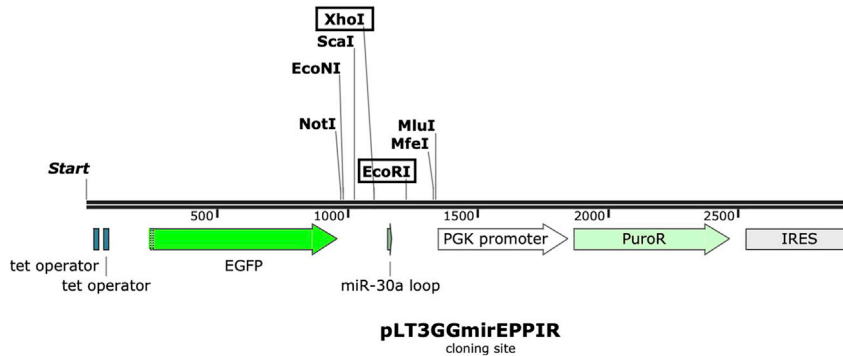


Figure 1. Map of pLT3GGmirEPPiR vector around the shRNA cloning site

Important elements are highlighted. For complete map refer to (Fellmann et al., 2013).

- b. Check for selection markers. In our case we have two: puromycin resistance and GFP.
- c. Check for available restriction sites that will be used in shRNA cloning. In our case we use *XhoI* and *EcoRI*.
- d. Check for antibiotic resistance genes for selection of transformed bacteria. In our case the resistance is ampicillin.

Library design and ordering

⌚ Timing: 2–3 weeks

We highly recommend using targeting oligonucleotides containing an optimized miR-E backbone. This results in a 97mer oligonucleotide per shRNA. We show here the process for assembling an shRNA library encompassing around 600 shRNAs.

Note: For biased targeted libraries like ours, we highly recommend including at least 20 shRNAs that will almost certainly elicit the predicted phenotype (positive control), as well as 20 shRNAs that will not (negative control). In our case, since we planned a drop-out screen, we included shRNAs targeting housekeeping genes as positive controls, and shRNAs targeting luciferase as negative controls.

2. 5 shRNAs were used per protein target. The miR-E shRNAs were selected by the online tool splashRNA (<http://splashrna.mskcc.org>) (Pelossof et al., 2017) and from Table S3 (Fellmann et al., 2013)(human genes).
 - a. For splashRNA use the default advanced parameters and only pick sequences with a score >1.0 (ideally >1.5).
 - b. Download the results to get the complete 97mer construct sequence.
 - c. For Table S3 filter out mouse genes, and again select the 97mer oligos.
3. After populating the shRNA list, order all 97mer oligonucleotides from IDT in Ultramer format (<https://eu.idtdna.com/pages/products/custom-dna-rna/dna-oligos/ultramer-dna-oligos>).
 - a. Item Type: 200 picomole Ultramer DNA plate Oligo.
 - b. Plate type: 384 Axygen Deep Well.
 - c. Concentration: 10 μ M.
 - d. Final volume: 20 μ L.
 - e. Purity/Services: RNase-free water.
4. Order the following primers:

- a. For the addition of *Xho*I, *Eco*RI restriction sites (HPLC purity):
 mirE_XhoI_F: TACAATACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG
 mirE_EcoRI_R: TTAGATGAATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCA
 Sequences in red are the respective restriction sites and sequences in blue bind to sequences found in all 97mers.
- b. For Sanger sequencing of the vectors (desalted purity):
 mirE_seq: TGTTTGAATGAGGCTTCAGTAC

Note: This sequence binds to the 5' end of the shRNA cloning site of the pLT3GmirEPIR vector. For other vectors the sequence would have to be adjusted.

- c. For the 1st PCR of library preparation (HPLC purity):
 1st_PCR_F:
 ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTGAAGCCACAGATGT*A
 1st_PCR_R: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAGAGATAGCAAGGTATT
 CA*G

Note: The asterisk denotes a thiophosphate modification at the 3' end meant to prevent primer degradation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
XL1-Blue Competent Cells	Agilent	Cat#200236
pLT3GEPIR-puro-shLuciferase	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Agarose NEEQ ultra-quality, 10 g	Carl Roth	Cat# 2267.1
Ethidium bromide solution 1%	Carl Roth	Cat#2218.1
GeneRuler 1 kb Plus DNA Ladder	Thermo Fisher Scientific	Cat#SM1332
EcoRI-HF	New England Biolabs	Cat#R3101
XhoI	New England Biolabs	Cat#R0146
Q5 Reaction Buffer Pack	New England Biolabs	Cat# B9027S
Q5 High-Fidelity DNA polymerase	New England Biolabs	Cat#M0491
dNTP-Set 1, 5 × 4 × 25 μmol (250 μL), 100 mM	Carl Roth	Cat#K039.2
Dimethyl sulfoxide	Merck	Cat#41640-M
Polyethylenimine, linear	Sigma-Aldrich	Cat#764604
GlycoBlue Coprecipitant (15 mg/mL)	Thermo Fisher Scientific	Cat#AM9516
CutSmart Buffer	New England Biolabs	Cat#B7204S
RNase A	Carl Roth	Cat#7156.1
Penicillin-Streptomycin - 100ML	Sigma-Aldrich	Cat#P4333
T4 DNA Ligase Buffer	New England Biolabs	Cat#B0202S
T4 DNA Ligase	New England Biolabs	Cat#M0202L
LB Broth	Thermo Fisher Scientific	Cat#10855001
Carbenicillin disodium salt, 25 g, plastic	Carl Roth	Cat#6344.3
Opti-MEM I Reduced Serum Medium	Thermo Fisher Scientific	Cat#31985047
Critical commercial assays		
GeneJET Gel Extraction Kit	Thermo Fisher Scientific	Cat#K0692
PureLink™ HiPure Plasmid Maxiprep Kit	Thermo Fisher Scientific	Cat#K210007
NEBNext® Multiplex Oligos for Illumina® (Dual Index Primers Set 1)	New England Biolabs	Cat#E7600S
NGS Fragment High Sensitivity Analysis Kit, 1–6,000 bp, 500 samples	Agilent	Cat#DNF-474-0500
NextSeq 500/550 High Output Kit v2 (75 cycles)	Illumina	Cat#FC-404-2005

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
HEK293TN	System Biosciences	Cat#LV900A-1; RRID:CVCL_UL49
Oligonucleotides		
mirE_XhoI_F: TACAATACTCGAGAA GGTATATTGCTGTTGACAGTGAGCG	This paper	N/A
mirE_EcoRI_R: TTAGATGAATTCTAGCC CCTTGAAGTCCGAGGCAGTAGGCA	This paper	N/A
mirE_seq: TGTTTGAATGAGGCTTCAGTAC	This paper	N/A
1st_PCR_F: ACACCTTTCCCTA CACGACGCTCTCCGATCTTAG TGAAGCCACAGATGT*A	This paper	N/A
1st_PCR_R: GTGACTGGAGTTCAGAC GTGTGCTCTCCGATCTAAGAGA TAGCAAGGTATTCA*G	This paper	N/A
Recombinant DNA		
psPAX2	Addgene	Cat#12260
pMD2.G	Addgene	Cat#12260
pLT3GEPIR	Fellmann et al., 2013	N/A
Software and algorithms		
SnapGene® software	Insightful Science	http://snappgene.com
FASTQ Generation software v1.0.0	Illumina	http://illumina.com
BCL Convert v2.0.0	Illumina	http://illumina.com
FastQC v0.11.3	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Bowtie v1.1.2	Langmead and Salzberg (2012)	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
SAMtools v1.3	Li et al. (2009)	http://www.htslib.org/
R v4.0.0	N/A	https://www.R-project.org/
Other		
Disposable syringe Omnifix With Luer-Lock fitting, 10 mL	Carl Roth	Cat# T552.1
Scalpel Cutfix sterile	Carl Roth	Cat#X004.1
Filtropur S 0,45 µm	Sarstedt	Cat#831826
5200 Fragment Analyzer System	Agilent	Cat# M5310AA
NextSeq 500 Sequencing System	Illumina	N/A (product discontinued)

Note: To align the shRNA sequences to the human genome, we used an older version of Bowtie (v1.1.2) and not the more current Bowtie 2, as the older version allows the use of a simple command that only allows perfect alignment (zero mismatches).

Alternatives: NextSeq 500 is discontinued, however the protocol can also be used with newer sequencing systems. We have used NextSeq 2000 (Illumina, Cat#20038897) with the same results.

MATERIALS AND EQUIPMENT

Prepare the following buffers for plasmid isolation.

Resuspension buffer		
Reagent	Final concentration	Amount
Tris-HCl pH 8.0 (1 M stock)	50 mM	2.5 mL
EDTA (0.5 M stock)	10 mM	1 mL
RNase A (10 mg/mL)	100 µg/mL	1:100 dilution, add to aliquot of buffer before use
ddH ₂ O	n/a	46.5 mL
Total	n/a	50 mL

Note: The RNase-free buffer can be stored indefinitely at 20°C–25°C. RNase A must be stored at –20°C and added shortly before use and used immediately, so RNase A should be added to an appropriate aliquot of the resuspension buffer.

Lysis buffer		
Reagent	Final concentration	Amount
NaOH (2 N stock)	0.2 N	10 mL
SDS (10% stock)	1%	10 mL
ddH ₂ O	n/a	80 mL
Total	n/a	100 mL

Note: The buffer can be stored indefinitely at 20°C–25°C.

The neutralization buffer is made by dissolving 29.45 g Potassium acetate powder in 100 mL ddH₂O, for a final concentration of 3 M. First dissolve the powder in 30 mL ddH₂O, calibrate the pH to 5.2 and then add ddH₂O to a final volume of 100 mL. The buffer can be stored indefinitely at 20°C–25°C.

STEP-BY-STEP METHOD DETAILS

Pooling of shRNAs and addition of *XhoI*, *EcoRI* restriction sites

⌚ Timing: 1 day

This step pools each individual shRNA in an equimolar mixture, which can then leverage the miR-E backbone to add the restriction sites necessary for cloning into the vector.

- Transfer 1 μL of each shRNA out of the 384-well plate into a 1.5 mL tube.
 - After all shRNAs have been pooled, dilute the 10 μM mixture to 330 nM (ca. 10 ng/μL) by adding 20 μL of the pool to 580 μL nuclease-free water.

Note: Any library size of pooled single-stranded DNA oligos can be safely amplified without the risk of underrepresenting or losing shRNAs.

- Store the original, undiluted shRNA pool at –80°C.
- Carry out the following PCR to add *XhoI*, *EcoRI* restriction sites. Setup at least 4 identical reactions. Include a negative control (no DNA template):

PCR reaction mix	
Reagent	Amount
DNA template (330 nM shRNA mix)	1 μL (= 10 ng ssDNA)
Q5 DNA Polymerase	0.5 μL
5× Q5 buffer	10 μL
mirE_ <i>XhoI</i> _F (10 μM stock)	2.5 μL
mirE_ <i>EcoRI</i> _R (10 μM stock)	2.5 μL
dNTPS (10 mM stock)	1 μL
DMSO	2.5 μL
ddH ₂ O	30 μL
Total	50 μL

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	25 s	8–10 cycles
Annealing	62°C	25 s	
Extension	72°C	20 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

- Take 10 μL out of the reaction, mix them with 2 μL 6 \times DNA loading dye and load all 12 μL on a 3% TAE/agarose gel.

Note: The PCR product is small (144 bp), so use an appropriate DNA marker. We recommend the GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, Cat#SM1332).

- Run the gel at constant 140 V for 30 min.
 - Inspect the gel for a band at around 140 bp corresponding with the expected PCR product (Figure 2). [Troubleshooting 1](#)

⚠ CRITICAL: Do not proceed with purification if overamplification (a second band above the expected PCR product) is visible.

- If no overamplification is visible, pool the PCR reactions in 1.5 mL tube and purify by ethanol precipitation.
 - Add 0.1 volume Na-Acetate pH 5.2, 2.5 volumes ice-cold 100% ethanol and 1 μL Glycoblue (15 mg/mL stock) to the sample.

Note: Volume refers to the initial volume of the PCR reactions, so if for example the PCR reaction is 50 μL , 0.1 volume means adding 5 μL Na-Acetate pH 5.2 and 2.5 volumes means adding 125 μL ethanol.

- Vortex the tube and incubate for at least 30 min at -20°C .
- Centrifuge for 30 min at 20,000 $\times g$ at 4°C .
- Carefully aspirate the supernatant with a 100–1,000 μL pipette.
- Add 700 μL 70% ethanol to the pellet.
- Centrifuge for 10 min at 20,000 $\times g$ at 4°C .
- Aspirate the supernatant and air-dry the pellets for 5 min.
- Resuspend pellet in 20 μL nuclease-free water and measure sample concentration and quality. Expected concentration is 500–1,000 ng/ μL and the 260/280, 260/230 ratios should be close to pure DNA (1.8 and 2.1, respectively).

⏸ Pause point: Samples can be safely stored at -20°C .

Restriction digest of vector and shRNA library

⌚ Timing: 1 day

In this step both the vector as well as the pooled shRNAs will be incubated with the XhoI and EcoRI restriction enzymes to generate sticky ends, facilitating their ligation.

- Setup at least 2 restriction digests for both the plasmid, as well as the pooled shRNAs (PCR product). Also include undigested controls (everything except enzymes) for both:

Digest mix	
Reagent	Amount
Plasmid / PCR product	10 µg / 5µg
XhoI	1.25 µL (25 U)
EcoRI-HF	1.25 µL (25 U)
CutSmart Buffer	5 µL
ddH ₂ O	Add to a total of 50 µL

7. Incubate the reactions for at least 90 min at 37°C.
8. Pool the reactions and load them on 1% and 3% TAE/agarose gels for the plasmid and PCR product, respectively.

△ **CRITICAL:** Ethidium bromide is used for TAE gels, which is carcinogenic.

△ **CRITICAL:** The TAE gels should have low amounts of ethidium bromide to minimize the risk of it intercalating with the DNA before purification. We recommend adding 2.5 µl ethidium bromide (10 mg/ml stock solution)/100 ml TAE buffer. Furthermore, if possible, adjust the UV lamp to the lowest possible intensity and expose the gels for the shortest duration possible to prevent damage to the DNA. [Troubleshooting 2](#)

9. Inspect the bands on the agarose gel.
 - a. The undigested PCR product should run slightly higher (at 144 bp) than the digested PCR product (at 130 bp).
 - b. The linearized digested plasmid should exhibit one clear unique band, while the supercoiled undigested plasmid should exhibit a double band that has progressed faster down the gel than its linearized counterpart.
10. After verifying the successful digest ([Figure 3](#)), excise the gel containing the digested bands using sterile scalpels.
 - a. Transfer the excised gel pieces into 2 mL tubes.
 - b. Proceed with extraction of DNA using the GeneJET Gel Extraction Kit, according to manufacturer's protocol A (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FMSG%2Fmanuals%2FMAN0012661_GeneJET_Gel_Extraction_UG.pdf).

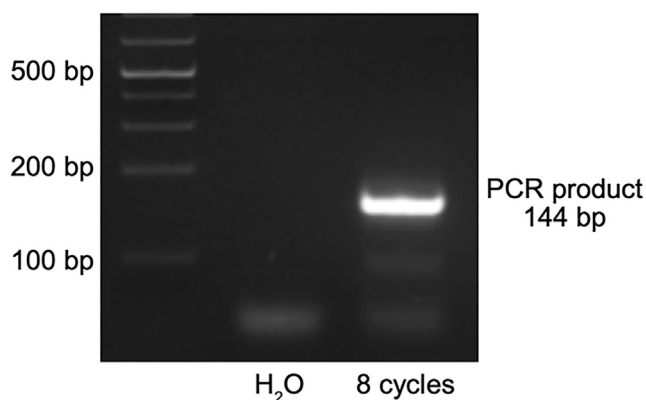


Figure 2. TAE/agarose gel image of PCR product after restriction site addition

No overamplification is visible after 8 PCR cycles. The H₂O negative control is appropriately empty.

- c. Carry out all protocol A (DNA extraction from the gel using centrifuge) steps including optional steps 4 and 6.

Note: Carry out all centrifugation steps at 20,000×g at 20°C–25°C in a table-top microcentrifuge.

- d. Elute the linearized plasmid and PCR product from the spin columns using 40 μL nuclease-free water. Repeat the elution once to increase DNA concentration.
e. Measure the concentration and quality of linearized plasmid and PCR product.

△ CRITICAL: For the linearized plasmid expect concentrations of at least 50 ng/μl, and for the PCR product around 5–10 ng/μl. Aim for at least 600 ng and 120 ng total DNA yield for plasmid and PCR product, respectively, before moving forward with the next step.

Note: Quality ratios (260/280, 260/230) are relevant only for the plasmid, as the low concentration of the PCR product perturbs these measurements.

▯▯ Pause point: Samples can be safely stored at –20°C.

Ligation of digested vector and PCR product

⌚ **Timing:** 2 days

This step brings the plasmid and PCR product containing complementary sticky ends together in a ratio that guarantees the cloning of all shRNAs in the pooled library.

△ CRITICAL: The PCR product:plasmid ratio is important to ensure that all shRNAs are cloned in the vector.

We highly recommend using a 15:1 molar ratio. To calculate the ratio, we recommend using the NEB ligation calculator (<https://nebiocalculator.neb.com#!/ligation>). For this particular reaction, the insert (digested PCR product) is 130 bp and the plasmid around 10 kb. A 15:1 ratio thus requires ca. 40 ng and 200 ng of PCR product and plasmid, respectively.

Note: This ligation reaction allows cloning of libraries of any size without the risk of underrepresenting or losing shRNAs.

11. Setup ligation reactions, as follows:

Ligation mix	
Reagent	Amount
Digested plasmid	200 ng
Digested PCR product	40 ng
10× T4 ligase buffer	2 μL
T4 ligase	1 μL (5 U)
ddH ₂ O	Add to a total of 20 μL

12. Incubate at 4°C–7°C (fridge) for 14–16 h.

Alternatives: Incubate the ligation reaction at 20°C–25°C for 60 min. We, however, recommend the 14–16 h incubation.

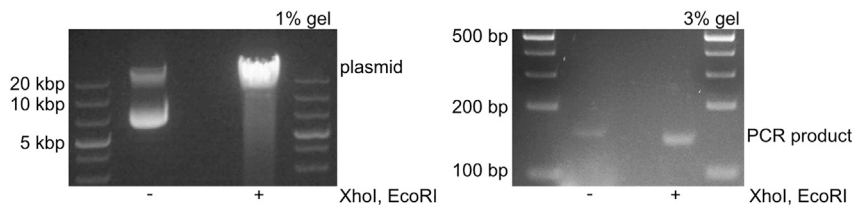


Figure 3. TAE/agarose gel images of plasmid (left) and PCR product (right) after restriction digest

Optional: After ligation is finished, heat inactivate the T4 DNA ligase by incubating at 70°C for 5 min.

- Proceed with the next step of the protocol without freezing the ligation reactions.

Transformation of competent cells and plasmid isolation

⌚ Timing: 1 week

In this step, we transform competent *E. coli* XL1-Blue cells (Agilent) with the ligation reactions, plate them on agar plates and then isolate the plasmid DNA. First, we carry out a small-scale transformation using one 10 cm plate to sequence 10–15 plasmids in order to check the ligation efficiency and the sequences of cloned shRNAs. Afterwards, we carry out the ligation in numbers that maintain a high representation of the shRNA library (>100-fold). For libraries comprising around 500 shRNAs, we recommend preparing fresh 15 cm culture plates (around 25–30 plates are required) with LB-agar and the antibiotic of choice for selection. In our case, we used 15 cm plates (30 plates in total) with carbenicillin (100 µg/mL) for around 600 shRNAs.

⚠ **CRITICAL:** Also plate untransformed bacteria, as a negative control, and bacteria transformed with the original undigested plasmid, as a positive control.

Alternatives: Instead of XL1-Blue bacteria, any competent bacterial strain can be transformed, such as XL10-Gold Ultracompetent Cells (Agilent, Cat.# 200314) or Agilent SURE2 Competent Cells (Agilent, Cat.# 200152).

- Thaw the XL1-Blue cells on ice.
- Transfer 50 µL to a 1.5 mL tube.
 - Add 6 µL of the ligation reaction to the cells.
 - Add 60 ng of the original undigested plasmid, as a positive control.
 - Add only water, as a negative control.
- Incubate on ice for 20 min.
- Heat shock the cells by placing the tubes for 45 s in a 42°C water bath.

⚠ **CRITICAL:** Do not exceed the duration of heat shock and proceed immediately to the next step.

- Incubate the cells for 2 min on ice.
- Add 800 µL LB medium to each tube.

Alternatives: Instead of LB medium, Super Optimal broth with Catabolite repression (SOC) medium (Thermo Fisher Scientific, Cat.# 15544034) can also be used.

Note: The medium should be at 20°C–25°C.

20. Incubate the tubes for 45 min at 37°C with shaking at 150 rpm.

Note: This step is necessary to allow the bacteria to start expressing the ampicillin resistance gene on the vector that they took up.

21. During this time pre-warm the LB-agar plates at 37°C.

22. Plate 100 μ L of transformed bacteria on each of the carbenicillin (100 μ g/mL) 15 cm culture plates.

23. Plate also the positive and negative control bacteria.

Note: Carbenicillin and ampicillin resistance are encoded by the same gene. We recommend carbenicillin as it prevents satellite colony growth.

24. Incubate all plates up-side-down for 16–18 h at 37°C.

25. On the next day, inspect the plates for colonies.

- a. The negative control plate must be empty.
- b. The positive control should have a very large number of colonies.

Note: First carry out the small-scale transformation to make sure that the quality of the pooled library is adequate. [Troubleshooting 2](#) and [3](#)

26. Pick out 10–15 individual colonies with a yellow pipette tip and transfer each into 14 mL round-bottom tubes containing 3 mL LB-medium with ampicillin (100 μ g/mL).

- a. Incubate for 16 h at 37°C with shaking.
- b. Transfer 1.5 mL from each culture into a 2 mL tube.
- c. Centrifuge at 20,000 \times g for 5 min at 20°C–25°C.
- d. Aspirate supernatant completely.
- e. Resuspend the pellet in 200 μ L resuspension buffer.

Note: Do not forget to freshly add RNase A to the resuspension buffer.

- f. Add 200 μ L lysis buffer.
- g. Mix by inverting the tube 5–10 times.
- h. Incubate for 5 min at 20°C–25°C.
- i. Add 350 μ L precipitation buffer and mix by inverting.
- j. Incubate for 3 min at RT.
- k. Centrifuge at 20,000 \times g for 5 min at 20°C–25°C.
- l. Transfer the supernatant to a fresh tube.
- m. Add 500 μ L isopropanol.
- n. Incubate for 15 min at –20°C.
- o. Centrifuge at 20,000 \times g for 5 min at 4°C.
- p. Discard supernatant and wash pellet with 500 μ L ice-cold 70% ethanol.
- q. Centrifuge at 11,400 \times g for 5 min at 4°C.
- r. Discard supernatant and air-dry the pellets for 5 min.
- s. Resuspend pellets in 30 μ L nuclease-free water.

Optional: Facilitate resuspension by incubating the tubes for 15–20 min at 37°C with shaking.

- t. Measure DNA concentration and quality.
- u. Check the shRNA sequences by Sanger sequencing, using the mirE_seq primer ([Figure 4](#)).

27. After validating that at least 80% of plasmids contain correct shRNAs, proceed with the large-scale transformation using 25–30 carbenicillin 15 cm culture plates.

- a. Carry out transformation as described above. Setup at least two ligation reaction reactions for a transformation of this scale.
- b. After colonies are clearly visible, add 1 mL LB-medium to each culture plate.
- c. Pool all cells using a sterile cell scraper into a 50 mL tube.

△ **CRITICAL:** Proceed immediately with plasmid isolation, do not continue bacterial culture.

28. Isolate plasmid DNA using the PureLink HiPure Maxiprep kit (Thermo Fisher Scientific, Cat.# K210007) with a slightly modified protocol.
 - a. Centrifuge the bacterial suspension at 11,400×g for 20 min at 4°C.
 - b. Discard the supernatant.
 - c. Add 10 mL resuspension buffer (R3 from kit).

Note: Do not forget to freshly add RNase A to the resuspension buffer.

- d. Add 10 mL lysis buffer (L7 from kit).
- e. Mix by inverting the tube. Do not vortex.
- f. Incubate for 5 min at 20°C–25°C.
- g. Add 10 mL precipitation buffer (N3 from kit).
- h. Mix by inverting until mixture is clearly homogeneous. Do not vortex.
- i. Incubate for 5 min at 20°C–25°C.
- j. Centrifuge at 11,400×g for 20 min at 20°C–25°C.
- k. During centrifugation, add 30 mL equilibration buffer (EQ1 from kit) to a HiPure Maxi column and allow the solution to drain by gravity flow.
- l. After centrifugation, place filter paper on the column and load the supernatant onto the column through the filter paper.

Note: DNA will pass through the filter paper.

- m. Add 60 mL wash buffer (W8 from kit) to the column. Discard the flow-through.
- n. Place a 50 mL tube under the column. Add 15 mL elution buffer (E4) to the column.

Note: The flow-through that ends up in the 50 ml tube is the eluted plasmid DNA.

- o. Add 10.5 mL isopropanol to the eluate and mix well.
- p. Incubate for at least 30 min on ice.
- q. Centrifuge at 11,400×g for 25 min at 4°C.
- r. Discard the supernatant.
- s. Wash the pellet with 5 mL ice-cold 70% ethanol.
- t. Centrifuge at 11,400×g for 15 min at 4°C.
- u. Remove supernatant and air-dry the pellet.
- v. Resuspend the pellet in 1–2 mL nuclease-free water.

Note: Incubate the pellet for around 30 min at 20°C–25°C to facilitate DNA resuspension.

- w. Measure plasmid DNA concentration and quality.
- x. Dilute the DNA sample to 1 µg/µL.

Note: Our 30 15 cm culture plate preparation yielded a total of 2.5 mg of highly-pure plasmid DNA.

▮▮▮ **Pause point:** Plasmid DNA can be safely stored at –20°C.

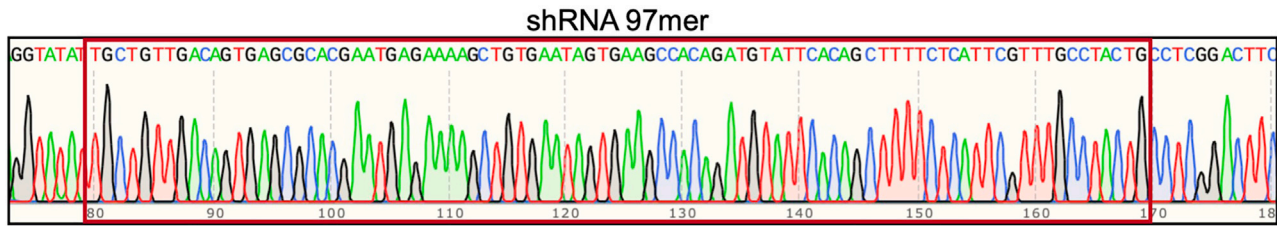


Figure 4. Chromatogram showing Sanger sequencing data of plasmid shRNA cloning site

The complete 97mer sequence is shown in the red box. Note that the base peaks are clearly distinct from each other indicating high DNA and sequencing quality.

Preparation of shRNA library for next-generation sequencing

⌚ Timing: 2 days

This step uses a small amount of the isolated plasmid DNA to generate a library suitable for Illumina sequencing. This is accomplished by two sequential PCRs that will specifically amplify the guide-strand of each cloned shRNA and will then add the index sequences necessary for multiplexed sequencing and the sequences required for hybridization of denatured libraries to Illumina NextSeq flowcells.

29. Setup the 1st PCR as follows. Also include a negative water control:

1st PCR reaction mix

Reagent	Amount
Plasmid DNA (1 µg/µL stock)	1 µL
Q5 DNA Polymerase	0.5 µL
5x Q5 buffer	10 µL
1st_PCR_F (10 µM stock)	2.5 µL
1st_PCR_R (10 µM stock)	2.5 µL
dNTPS (10 mM stock)	1 µL
DMSO	2.5 µL
ddH ₂ O	30 µL
Total	50 µL

1st PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	2 min	1
Denaturation	98°C	10 s	8–10 cycles
Annealing	50°C	20 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

30. As with step 3, take 10 µL of the PCR reaction, add 2 µL of 6x DNA loading dye and load all 12 µL on a 3% TAE/agarose gel.

31. Inspect the gel. A single band at around 188 bp is expected. Make sure no overamplification is visible (Figure 5). [Troubleshooting 1](#)

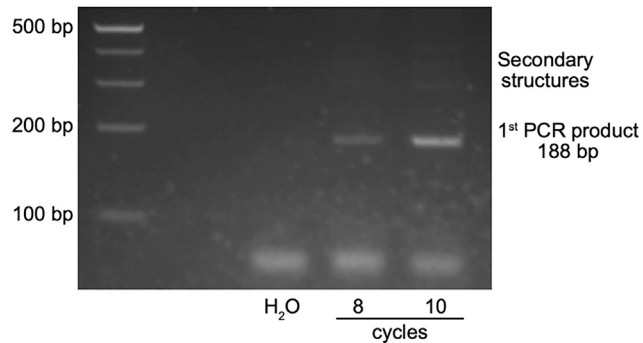


Figure 5. TAE/agarose gel image of 1st PCR product

The PCR product displays faint secondary structures after 10 amplification cycles and will thus be discarded. The 8 cycle PCR product will be purified and used further. The water control is appropriately empty.

32. Purify the PCR product without secondary structures with ethanol precipitation as described in step 5.

Alternatives: Gel extraction of the 188 bp band is also possible, but it will result in significantly reduced DNA yield and quality.

33. Measure the DNA concentration and quality.
34. Dilute the sample to 1 ng/ μ L.

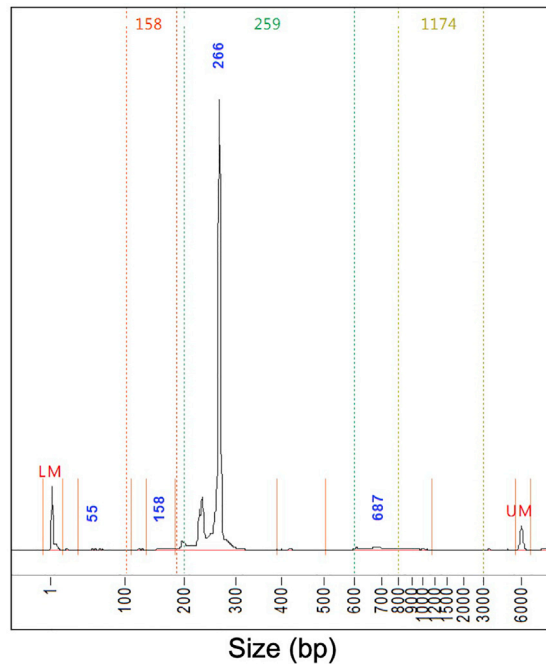
△ CRITICAL: All subsequent steps need to be carried out in a post-PCR workspace to avoid contaminating other workspaces with indexed samples.

▯▯ Pause point: Samples can be safely stored at -20°C .

35. Setup the 2nd PCR as follows:

2 nd PCR reaction mix	
Reagent	Amount
Purified 1 st PCR product (1 ng/ μ L)	1 μ L
Q5 DNA Polymerase	0.5 μ L
5 \times Q5 buffer	10 μ L
NEBNext_i5_index_primer (10 μ M stock)	2.5 μ L
NEBNext_i7_index_primer (10 μ M stock)	2.5 μ L
dNTPS (10 mM stock)	1 μ L
ddH ₂ O	32.5 μ L
Total	50 μL

2 nd PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98 $^{\circ}$ C	2 min	1
Denaturation	98 $^{\circ}$ C	10 s	15 cycles
Annealing	65 $^{\circ}$ C	20 s	
Extension	72 $^{\circ}$ C	30 s	
Final extension	72 $^{\circ}$ C	5 min	1
Hold	4 $^{\circ}$ C	forever	



Smear Analysis

100 bp to 185 bp	0.0165 ng/ul	0.1725 nmole/L
200 bp to 600 bp	1.2092 ng/ul	7.6723 nmole/L
800 bp to 3000 bp	0.0098 ng/ul	0.0138 nmole/L

Figure 6. Fragment analyzer image and smear analysis of a pooled shRNA library, suitable for next-generation sequencing

The sample shows a sharp peak around 260 bp, which was the size of the 2nd PCR product. Index primer (100–185 bp) or secondary structure contamination (800–3,000 bp) are appropriately low. LM, UM = lower and upper marker.

Note: Setup at least two 2nd PCRs.

36. Load the entire reaction to a 3% TAE/agarose gel.
 - a. Inspect the gel and excise the band at around 250 bp (expected size is 257 bp).
 - b. Carry out gel extraction as described in step 10.
37. Load the DNA library on a parallel capillary electrophoresis system. We used a Fragment Analyzer system (Agilent)
 - a. Load samples according to manufacturer's protocol (<https://www.agilent.com/cs/library/usermanuals/public/quick-guide-dnf-474-hs-ngs-fragment-kit-SD-AT000134.pdf>).
 - b. After electrophoresis, assess the concentration and quality of the library with smear analysis (Figure 6). [Troubleshooting 4](#)

▮▮▮ **Pause point:** Samples can be safely stored at -20°C (separate post-PCR sample storage).

Sequencing and analysis of shRNA library

⌚ **Timing:** 3 days

38. Sequence the library using an Illumina sequencing system according to manufacturer's protocol. We used the Illumina NextSeq 500 sequencing system.

- a. Prepare samples according to manufacturer's protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/nextseq/nextseq-500-system-guide-15046563-07.pdf).

△ **CRITICAL:** Sequence for at least 41 cycles.

Note: Sequencing can be done as single-read (SR) or as paired-end (PE) sequencing. However, for all analysis steps, only the generated "read1" is required. The length of read1 has to be at least 41 cycles (= 41 bp of the template sequence). The first 19 bp sequenced correspond to the invariable shRNA-loop sequence used for binding of the first-step PCR primer "1st_PCR_F". The following 22 bp correspond to the variable sequence of the shRNA guide-stem.

39. Analyze the shRNA distribution and generate plots as follows:
 - a. Demultiplexing and FASTQ generation directly via the Illumina Base Space Sequencing Hub using the tool FASTQ Generation software v1.0.0 or BCL Convert v2.0.0.
 - b. The sample-specific FASTQ files are used for a Bowtie alignment to a reference dataset comprising all initially cloned shRNA sequences. Counting of all alignments per recovered and sequenced shRNA allows to calculate and visualize their distribution (frequencies) within the library.

△ **CRITICAL:** Make sure that the programs `bowtie1`, `samtools` and `R` are installed in a linux environment.

Note: The steps shown below are applicable to any kind of shRNA screening sample, provided that guide-strand recovery from plasmid DNA was done according to the above outlined steps.

40. Generate a reference library for Bowtie alignments
 - a. Generate a table in FASTA format that contains the guide-stem sequences of all shRNAs that were cloned and are expected to be represented in the pooled library.
 - b. Use this table to build a `bowtie1` compatible "reference genome":

```
>bowtie-build table.fasta reference_name
```

41. Align the sequence raw data (in FASTQ format) to the reference genome using `bowtie1`. In case of a paired-end sequencing approach, only use the sample-specific FASTQ files containing `read1` for the alignment and write the alignment-output into a `sam`-file.

```
>bowtie -v 0 -m 1 -5 19 -best -S /path-to-  
reference/reference_name input.fastq output.sam
```

Note: The option "`-v 0`" in the command does not allow any mismatches during alignment. The option "`-m 1`" in the command reports only reads with one valid alignment. The option "`-5 19`" specifies that the first 19 bp are ignored for the alignment to the reference genome.

Note: If `read1` is longer than 41 bp, the additional option "`-3 xx`" has to be used in the alignment command to ignore the `xx` bp from the 3'-end of the read that do not belong to the guide-stem.

42. Convert the output.sam file into a bam-file

```
>samtools view -bS output.sam output.bam
```

43. Count the number of reads aligned to each entry in the reference dataset

```
>samtools view output.bam | cut -f 3 | sort | uniq -c >
counts_per_shRNA.txt
```

44. Convert the generated "counts"-file into a comma-separated-value (.csv) table that consists of rows corresponding to all individual shRNAs in the library and of columns containing the counts per shRNA identified in the sequenced sample(s). For this step, either Microsoft Excel or R can be used.

45. Normalize the raw counts per shRNA by calculating CPM values (counts per million). This is achieved by applying the following formula to every individual raw count value of a sample:

$$(\textit{individual raw count} \div \textit{column sum count} \times 1,000,000) = \textit{individual CPM value}$$

46. Read the CPM normalized counttable into R and generate a histogram to plot the distribution of shRNAs within the library:

```
>counttable=read.csv(file="complete-path-to-
table/counttable.csv", header=TRUE, row.name=1)
>hist(log10(counttable$CPM_values), breaks=100,
border="blue", xlim=c(2,6), ylim=c(0,60),
xlab="log10(number of counts per million (CPM))")
```

47. Inspect the generated plot and the distribution of shRNAs in the library ([Figure 7](#)). [Troubleshooting 5](#)

Packaging of shRNA library plasmids into lentiviruses

⌚ Timing: 4 days

In this step HEK293TN cells are transfected with the shRNA plasmid library along with two packaging plasmids required for the production of lentiviruses encompassing the shRNA library. In our case, we used the psPAX2 and pMD2.G packaging plasmids.

Alternatives: HEK293TN cells are optimized for lentivirus production, however the parental HEK293 cells (CLS, Cat# 300192) can also be used. Additionally, the African green monkey COS-7 kidney cells (ATCC, Cat.# CRL-1651) and human TE671 rhabdomyosarcoma cells (CLS, Cat.# 300355) can be used, albeit with lower expected virus titers than the HEK293TN cells ([Marino et al., 2003](#)).

48. Count HEK293TN cells and seed 5 million cells in a 10 cm culture plate.

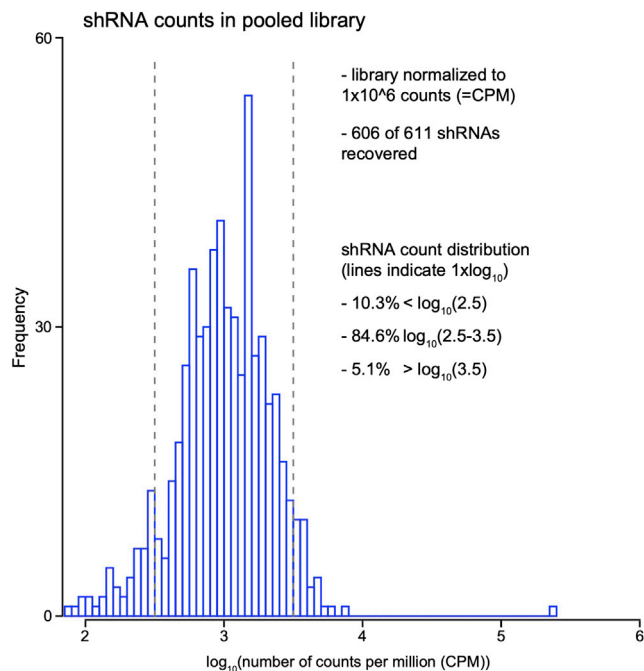


Figure 7. Histogram of normalized counts showing the number and distribution of shRNAs in the sequenced library

Note: Make sure to spread the cells evenly across the culture plate.

- Let the cells grow for 14–16 h.
- Prepare the transfection mixtures in two 1.5 mL tubes as follows:
Transfection mixes/ 10 cm culture plate

DNA mix	PEI mix
500 μL Opti-MEM	500 μL Opti-MEM
10 μL shRNA plasmid library	30 μL PEI
10 μL psPAX2 (1 $\mu\text{g}/\mu\text{L}$ stock)	
2.5 μL pMD2.G (1 $\mu\text{g}/\mu\text{L}$ stock)	

Note: Avoid prolonged exposure of PEI to light sources.

- Mix both mixes by pipetting up and down.
- Incubate for 5 min at 20°C – 25°C .
- Add the complete DNA mix to the PEI mix drop-wise.
- Mix by pipetting up and down. Do not vortex.
- Incubate for 20 min at 20°C – 25°C .
- Meanwhile, aspirate the growth medium from the HEK293TN cells and wash once with PBS.
- Add 5 mL transfection medium (medium supplemented with 2% FBS, no antibiotics).
- After incubation is finished, add the transfection mix dropwise to the cells and gently swirl the plates.

△ CRITICAL: After this point lentiviruses are being produced and thus cell culture needs to continue in a biosafety level 2 compliant facility.

- k. 8 h after transfection, aspirate transfection medium, wash with PBS and add 6 mL full medium to the cells.
- l. Harvest the lentiviruses 24 and 48 h after transfection. [Troubleshooting 6](#)

Note: Virus production peaks at 48 h and then rapidly decreases. Any further harvests would just dilute the virus titer.

- m. Collect the virus-containing culture medium with a 10 mL syringe.
- n. Filter the medium through a 0.45 μm filter.
- o. After the second harvest, aliquot the virus in 15 mL tubes.
- p. Directly transduce the cells of choice.
- q. Alternatively, flash-freeze the tubes and keep the viruses at -80°C .

EXPECTED OUTCOMES

This protocol will result in high-quality shRNA libraries cloned in a doxycycline-inducible vector, allowing the stable and inducible expression of the shRNAs in any available cell line. We expect the generated libraries to encompass almost all of the originally pooled shRNAs (606/611 recovered in our final library – 99% recovery) with low standard deviation in shRNA count distribution (84.6% of shRNA counts within $\log_{10}2.5\text{--}3.5$ range). Finally, 83% of our sequenced library exhibited zero mismatches during alignment ([Figure 7](#)).

This library can then be rapidly packaged into lentiviruses for stable transduction of a cell line of choice with the steps outlined here. We expect an optimal virus titer thanks to the use of HEK293TN cells and the restriction of the virus supernatant harvest to 24 h and 48 h after transfection.

We also note that this protocol can be utilized to clone individual shRNAs, which will most likely be necessary for the validation of hits derived from the screen.

LIMITATIONS

This protocol, while streamlined, still consists of numerous long and laborious steps. The first step of pooling the individual shRNAs is a prime example, as each shRNA needs to be transferred into one 1.5 mL tube. As missteps will carry over, the protocol includes several quality control steps, such as overamplification checks and Sanger sequencing of individual colonies to ensure the generation of high-quality libraries.

The transformation step can be limiting; genome-wide libraries would require very large numbers of transformed bacteria and agar plates to ensure a high representation of all shRNAs. Despite that, genome-wide screens can be carried out, albeit with a lower representation.

Finally, while the optimized miR-E shRNAs result in around 80% knock-down of target proteins in our hands, this depletion is still not comparable to sgRNAs, which should theoretically elicit a complete knock-out effect. Therefore, we do not recommend generating shRNA libraries targeting factors whose knock-down would not result in a clear phenotype. In any other case, shRNA libraries are a viable, cheaper and simpler alternative to sgRNAs.

TROUBLESHOOTING

Problem 1

Steps 4 and 31.

Overamplification of PCR product visible as double band on TAE/agarose gels.

Potential solution

Overamplification arises from excessive PCR amplification cycles. Keep the number of PCR cycles low. In our experience, 8–10 cycles are enough to generate a faint band on the TAE/agarose gel at the correct size.

Problem 2

Steps 8 and 25.

Point mutations in shRNA 97mer sequence after Sanger sequencing of individual colonies.

Potential solution

We highly recommend specifically using Q5 polymerase for all relevant amplification steps and not other *Taq* counterparts. The amplification fidelity is approximately 280× higher than *Taq* (<https://international.neb.com/products/m0491-q5-high-fidelity-dna-polymerase#Protocols,%20Manuals%20&%20Usage>). Additionally, use low ethidium bromide concentrations in all TAE/agarose gels and keep UV light exposure to a minimum. Always work with fresh, sterile scalpels for gel band excision.

Problem 3

Step 25.

Large proportion of empty vectors after Sanger sequencing of individual colonies.

Potential solution

Optimize ligation ratios between PCR product and vector. We have had best results with a 15:1 ratio, however 5:1 to 10:1 can also be tested.

Problem 4

Step 37.

Index primer, secondary structure contamination of shRNA library.

Potential solution

Repeat the 2nd PCR. Set up more reactions and pool them before gel purification. Pooling will increase the end concentration and quality of the DNA library.

Problem 5

Step 47.

High standard deviation in shRNA count distribution.

Potential solution

Avoid gel purifications of PCR unless explicitly stated in the protocol, particularly in the first steps of the protocol (restriction site addition). Ethanol precipitation will generate markedly purer and more concentrated DNA.

Problem 6

Step 48.

Low transfection efficiency of HEK293TN cells.

Potential solution

Make sure that the cells are around 70%–80% confluence and spread evenly on the culture plate at the time of transfection. Keep PEI away from light and always add the mixes drop-wise, since the PEI-DNA polymers are delicate. Make sure the transfection medium does not contain antibiotics and do not exceed 8 h of transfection, as PEI is toxic to cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dimitrios Papadopoulos (dimitrios.papadopoulos@uni-wuerzburg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all code generated during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, D.P., C.P.A., and M.E.; investigation, D.P. and C.P.A.; writing – original draft, D.P. and C.P.A.; writing – review & editing, D.P.; funding acquisition, M.E.; supervision, M.E.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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