transEDIT gRNA Pool Cloning Guide

Vectors: pCLIP-gRNA and pCLIP-ALL

Format:

Vial A (125 µL): PCR amplified oligo pool- Prepared for Gibson Assembly Reaction (15 ng/µL).

Vial B (100 µL) Vector Backbone- Prepared by restriction digest and CIP treatment, column purified (200 ng/µL).

This guide provides information for the cloning of transEDIT CRISPR gRNA lentiviral pooled libraries in gRNA plus Cas9 expression vectors (pCLIP-ALL) and transEDIT lentiviral gRNA (pCLIP-gRNA) vectors.

Section 1: Product specifications

Storage

Plasmid components are shipped on dry ice and should be stored at -80°C upon receipt.

Components

- Vial A (125 µL): PCR amplified oligo pool- Prepared for Gibson Assembly Reaction (15 ng/µL).
- Vial B (100 µL) Vector Backbone- Prepared by restriction digest and CIP treatment, column purified (200 ng/µL).

Note: All references to "negative control" are the backbone-only reactions from the Gibson assembly.

Library Specifications

	Human WG gRNA	Mouse WG gRNA	Human WG pCLIP-ALL	Mouse WG pCLIP-ALL
Species	**Homo sapiens**	**Mus musculus**	**Homo sapiens**	**Mus musculus**
Number of Targeted Genes	**18,972**	**19,674**	**18,972**	**19,674**
Targeting Constructs/ Gene	**4**	**4***	**4**	**4***
Control (non-Targeting sgRNAs	**1,000**	**1,000**	**1,000**	**1,000**
Total sgRNA Constructs	**76,888**	**79,633**	**76,888**	**79,633**

**A few genes have only 3 sgRNA targeting the gene.*

Section 2: Cloning Workflow

Gibson Assembly:

Required Reagents

5x Isothermal Buffer

[Final]	Amount	Stock (reagent)
25% PEG-8000	0.75 g	
500 mM Tris-HCL pH 7.5	1.5 mL	1 M
VIAL50 mM MgCl2	75 µL	2 M
50 mM DTT	150 µL	1 M
1 mM dATP	30 µL	100 mM
1 mM dTTP	30 µL	100 mM
1 mM dCTP	30 µL	100 mM
1 mM dGTP	30 µL	100 mM
5 mM NAD	300 µL	50 mM
H2O	up to 3 mL	
Total	3 mL	

1.33x Taq Ligase Gibson Assembly Master Mix

Component	Amount
Taq Ligase	25 µL
5x isothermal buffer	100 µL
T5 exonuclease	2 µL
Phusion polymerase	7 µL
Nuclease-free water	236 µL
100x NAD+	5 µL
Total	375 µL

Material	Co.	Catalog number
Phusion HiFi DNA Polymerase	NEB	M0530S/L
Taq DNA Ligase	NEB	M0208S/L
T5 Exonuclease	NEB	M0663S/L
NAD+ 100x	NEB	B9007S

Gibson Procedure:

1. Set up Gibson Assembly reaction according to **Table 1.**
2. Aliquot 20 µl reaction in PCR tubes.
3. Incubate tubes at 50°C for 1 hour.

Table 1: Gibson Reaction Components

Component	Amount per reaction	Final concentration
Taq Gibson master mix, 1.33 x	15 µl	1×
Linearized plasmid backbone	1.67 µL	16.5 ng/µl
PCR amplified oligo pool	3.33 µL	2.5 ng/ µl
Total	20 µl	

Recommendations:
A minimum of 4 Gibson reactions performed per pool
At minimum perform 1 Gibson reaction per 5,000 oligos (For the WG libraries this will equate to performing 16 Gibson reactions)

DNA precipitation

Procedure

1. Carefully transfer DNA samples to a clean tube suitable for precipitation (e.g 1.5mL microcentrifuge tube).
2. Add 1/10 volume 3M NaOAc (pH5.2), 1 volume 100% isopropanol (room temperature) and 1% Glycoblue™ coprecipitant (ThermoFIsher). Vortex to mix and incubate at room temperature for 15 minutes.
3. Centrifuge at ≥ 15,000 x g for 20 min at 4°C.
4. Carefully remove supernatant (precipitated DNA should appear as a small light blue pellet at the bottom of the tube.
5. Add 2.0 mL 70% ethanol and vortex to resuspend the pellet (only use 1.0 mL if using 1.5 mL tube).
6. centrifuge at ≥ 15,000 x g for 10 min at 4°C.
7. Carefully remove supernatant and air-dry pellet for 10 minutes.
8. Resuspend DNA in TE buffer (prewarmed to 55°C). Use 5 µl TE buffer per each Gibson assembly reaction performed. Incubate DNA at 55°C for 10 minutes to ensure complete resuspension.
9. Combine DNA from all reactions.
10. Determine DNA concentration.

Cell Transformation and Colony Selection

Required Reagents

LB + Carbenicillin (LB-C)

Component	Amount
LB Broth - Teknova	23 g
dH2O	900 mL
Carbencillin (100µg/mL)	1.8 mL

1. Add LB broth to dH20.
2. Stir solution.
3. Autoclave.
4. Once solution cools, add Carbenicillin.

LB medium + Carbenicillin + glycerol (LB-C-G)

Component	Amount
LB Broth - Teknova	23 g
dH2O	828 mL
Glycerol	72 mL
Carbencillin (100µg/mL)	1.8 mL

1. Add LB broth to dH20.
2. Stir solution.
3. Add glycerol and swirl until thoroughly mixed.
4. Autoclave.
5. Once solution cools add Carbenicillin.

For cell transformation we recommend using Endura Cells with electroporation. MA133 Endura Competent Cells (bigcontent.io).

It is highly recommended that a "test" electroporation be performed initially using only 1 pool DNA and 1 negative control DNA to verify that the cloning was successful, and that electroporation efficiency is adequate (to reach desired coverage) and to go forward with the remaining pool DNA.

Additional Recommendations

1. 1 electroporation per 5,000 oligos of library.
2. Include Gibson Assembly/Ligation negative control.

Cell Plating

Plating Cells on LB plates	Recommended QC Step
1. Pre-warm large (24.5 x 24.5 cm square) LB Agar Plates with Carbenicillin (LB-C) plates at 37°C. 2. Add 1 ml recovery medium to each original reaction to total 2 ml. 3. Plate 2 ml medium onto each large LB-C plate. 4. Spread the liquid culture until it is largely absorbed into the agar and won't drip when turned upside down. 5. Incubate plates inverted overnight at 30°C.	1. Pre-warm standard (10 cm diameter) LB-C plates at 37°C. 2. Dilute Transformed Cells (for each electroporation reaction). a. Dilution 1: 3uL cells in recovery medium into 297uL medium (1:100) b. Dilution 2: 11uL of Dilution 1 cells in 99uL medium (1:10) dilution 6. Plate 100 µL cells on to standard LB-C plate. 7. Plate 100 µl negative control sample onto a standard LB-C plate. 8. Incubate plates inverted overnight at 30°C. 9. Count positive and negative colonies. 10. Determine positive to negative colony ratio. 11. Determine the total number of colonies to number of constructs ratio.
	NOTE: It is recommended that colonies on the negative plate are <5% of the number of colonies on the positive plate and that there is a 50x coverage of clones to constructs. If these parameters are not met a new cell transformation should be performed.

Harvest colonies from the LB agar plates and DNA Prep

1. Pipette 10.0 mL LB-C-G medium onto each LB agar plate.
2. Gently scrape the colonies off with a cell spreader and transfer the liquid with scraped colonies into sterilized 500mL Erlenmeyer flasks.
3. For each LB agar plate, repeat Step 1 and Step 2 for a total of 2 LB-C-G medium washes to capture any remaining bacteria (e.g. the total volume for 16 plates ~320mL).
   After this step It is acceptable, although not ideal, to freeze the collected bacterial slurry at -80°C for later use.
4. To 8 4L Erlenmeyer flasks add 770mL LB+C medium. Add 30mL of the plate slurry to each flask bringing the total volume to 800mL.
5. Incubate liquid culture in shaking incubator for 4 hours (32°C, 220rpm).
6. Following the incubation use 4 Gigapreps (Qiagen) to purify the DNA.

Critical Step *Using an endotoxin-free plasmid purification kit is important at this step for avoiding endotoxicity in virus production and mammalian cell culture. To ensure that the plasmid preparation is endotoxin-free.*

Appendix 1 – Vector information

The **transEDIT™ pCLIP-ALL and pCLIP-gRNA expression vectors** allow transient and stable transfection; as well as the stable delivery of a gRNA into host cells via a replication-incompetent lentivirus. The **pCLIP-ALL expression vectors** allow efficient delivery of gRNA and Cas9 in one vector. The **pCLIP-gRNA expression vectors** are designed to be used in conjunction with a separate Cas9 expression vector or cells that already express Cas9.

Vector Features:

- Vector selection options (**Figure 1**):
  - Promoter selection (EFS or hCMV) to tailor to your cell line
  - Antibiotic selection (puromycin, hygromycin, or blasticidin) for enrichment of transduced cells
  - Or fluorescent marker (Green or RFP) for direct detection of expression
- Transduction of primary and non-dividing cells
- Cas9 vectors are available separately for use with the pCLIP-gRNA vectors

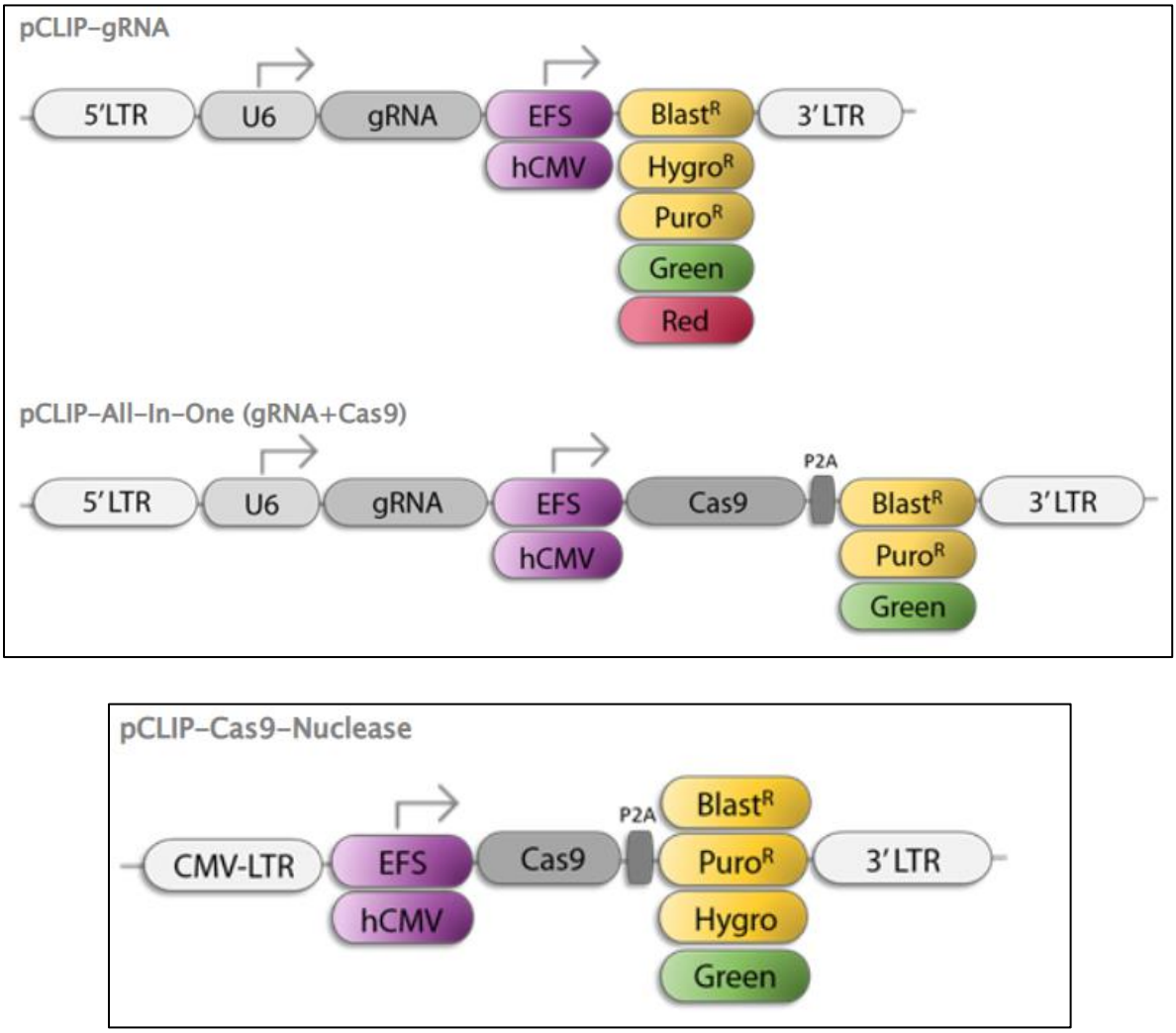


Figure 1. Schematic depicting options for the pCLIP-gRNA and pCLIP-Cas9-nuclease vectors

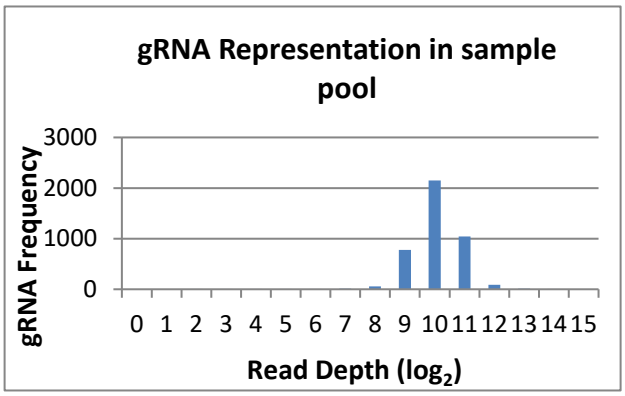
www.transomic.com 866-833-0712 support@transomic.com

Full vector sequences and maps are available at https://www.transomic.com/cms/Product-Support/Vector-Maps-and-Sequences/CRISPR-Vector-Maps.aspx

gRNA Representation in Pooled Libraries-Quality Control

We strongly recommend that NextGen Sequencing be performed after cloning to confirm the oligo representation in the final library.

CRISPR gRNA pools should be developed using strategies that maintain equimolarity of the pooled gRNA constructs – to ensure that screening results are not artificially biased to any one or some gene(s) or construct(s) prior to the screen. Evaluation of all gRNA/Cas9 plasmid DNA pools by next-generation sequencing (NGS) for distribution and representation analysis is recommended. An example representative graph of gRNA distribution is shown in **Figure 2.** When the plasmid DNA pools are equimolar, the virus produced from these should have the same equimolar representation of gRNAs.



Pool Screening Metrics	
Total gRNA in Pool	5080
gRNA Detected (by NGS)	5004
% Recovery	98.5%
# ± 5X Mean Read Depth	4737
% ± 5X Mean Read Depth	94.7%

Figure 2. Example histogram showing distribution of gRNA from a plasmid DNA pool as seen by NGS analysis (left). Summary statistics of QC analysis (right). The summary includes the total gRNA constructs pooled to create the library and the number detected by NGS.

Appendix 2 - PCR and NGS sequencing primer sequences pCLIP-ALL and pCLIP-gRNA

Primary NGS Forward (5'->3')
GTACCGAGGGCCTATTTCCCATG

Primary NGS Reverse (5'->3')
GACTCGGTGCCACTTTTTCAAGTTG

Crispr Secondary PCR Forward primer (5'->3')
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGCCTATTTCCCATGATTCC

CRSP-primary-F	CRSP-primary-F	gtaccgagggcctatttcccatg
CRSP-primary-R	CRSP-primary-R	gactcggtgccacttttttcaagttg
CRISPR-Secondary-F	CRSPR-F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGCCTATTTCCCATGATTCC

CRISPR-Secondary-R-Index 2	CRSPR-R Index #2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 4	CRSPR-R Index #4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 5	CRSPR-R Index #5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 6	CRSPR-R Index #6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 7	CRSPR-R Index #7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
CRISPR-Secondary-R-Index 12	CRSPR-R Index #12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACTTTTTCAAGTTGATAACGG
Custom Read 1 sequencing primer	CRSPR_NGS Read 1	CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

If low level multiplexing is needed (6 samples or less), please refer to *Multiplexing Sample preparation Guide (Illumina® Part# 1005361)* for recommendations on which sets of the indexes above can be used together.

Appendix 3 – References and recommended reading

Transomic Product guides and technical support documents:
https://www.transomic.com/cms/Product-Support/FAQs/CRISPR-FAQs.aspx
https://www.transomic.com/cms/Product-Support/Product-Guides/CRISPR-Product-Guides.aspx

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