CLUE Wetlab Pipeline SOP

Contents:

Introduction:

CLUE is a bioinformatics and wet-lab pipeline, designed for fast and efficient cloning of multiple custom sgRNA libraries from a single oligonucleotide pool. This protocol describes the wet-lab cloning part, which requires the user to already have an oligonucleotide pool, designed with the CLUE webtool, ready to use. For the basic principle of the CLUE pipeline please refer to the corresponding publication.

Materials:

Reagent	Vendor and Cat. Number
Zero Blunt™ TOPO™ PCR Cloning Kit, without competent cells	451245, Invitrogen
Endura Competent cells	MA133, Endura oder BioCat 60242-1-LU
Kapa Hifi Polymerase	Roche, NC0580933
GlycoBlue Co-Precipitant	Thermo Scientific, AM9515
FastDigest BpiI	Thermo Scientific, FD1014
NEBuilder Hifi Master Mix	New England Biolabs, E2621L
NucleoSpin Gel and PCR Clean-Up Kit	Macherey-Nagel,
NucleoBond Xtra Maxi Kit	Macherey-Nagel,

Protocol:

Part 1: Oligonucleotide pool amplification and sub-cloning (*optional, but highly recommended*)

1a) Oligonucleotide pool amplification by PCR

- Oligopool DNA is usually received lyophilized
- Resuspend oligopool DNA in TE buffer at a final concentration of 10 ng/µl
  Note: lyophilized and resuspended oligopool DNA can be stored at -20°C for several months
- Amplify the entire oligopool DNA by PCR using primers Pool_ampl_f and Pool_ampl_r (PCR 1) in the following setup:

Component	**Amount**
Oligopool DNA	10 ng* (1 µl)
Primer Pool_ampl_f (10 µM stock)	0.75 µl
Primer Pool_ampl_r (10 µM stock)	0.75 µl
5X Kapa Hifi Fidelity Buffer	5 µl
dNTP mix (10 mM each)	0.75 µl
Kapa Hifi Polymerase (1 U/µl)	0.5 µl
Water to total volume of 25 µl	16.25 µl

*Note: with an oligo length of 150 nt, 10 ng of DNA amount to >6x10^{10} oligos, which should be sufficient coverage for the vast majority of oligonucleotide pools

- Run the PCR as follows:

3 min	98°C	
30 sec	98°C	10 – 15 cycles (the fewer the better, might be subject to optimization)
30 sec	62°C	
15 sec	72°C	
2 min	72°C	
forever	4°C	

1b) TOPO cloning of amplified oligonucleotide DNA

- Amplified DNA can directly be used for TOPO cloning (ZeroBlunt TOPO PCR Cloning Kit, Invitrogen), no clean-up is necessary
- Set up the TOPO reactions as follows:

Component	**Amount**
PCR reaction	2 µl
Water	2 µl
Salt Solution	1 µl
TOPO Vector	1 µl

- Perform one TOPO reaction per 5000 oligos within your pool (i.e. for a pool with 15,000 different oligos perform 3 reactions)
- Incubate at room temperature for 30 min
- Bring the volume of each reaction to 100 µl with water
- To each reaction add

  - 100 µl isopropanol
  - 2 µl 5M NaCl
  - 1 µl GlycoBlue Co-Precipitant
- Vortex samples thoroughly
- Incubate at room temperature for 15 min
- Centrifuge at >16,000 x g for 15 min
- Discard supernatants
- Wash pellets twice with 70% Ethanol
- Air-dry pellets and Resuspend each pellet in 2 µl water

1c) Electroporation of TOPO-cloned oligonucleotide pool

- Thaw Endura electrocompetent bacteria (Lucigen) on ice
- Aliquot 25 µl thawed bacteria into pre-chilled tubes
- Add 2 µl TOPO-cloned oligonucleotide pool from previous step and gently mix by stirring with the pipette tip
- Transfer bacteria-DNA suspension to pre-chilled electroporation cuvettes (1.0 mm)
- Electroporate with the following settings:
  - 25 µF
  - 600 Ω
  - 1800 V
- After electroporation, immediately add 975 µl Recovery Medium (Lucigen) and transfer the cell suspension to a fresh culture tube
- Incubate bacteria at 37°C and 250 rpm for 1 hour
- *optional*: Take 10 µl cell suspension for further dilution steps to determine the titer and electroporation efficiency
- Plate bacteria on LB agar containing 50 µg/ml kanamycin on 245 mm squared dishes
- Let plates dry at room temperature and then incubate at 37°C over night
- Float-off all bacterial colonies grown with LB medium containing 50 µg/ml kanamycin and collect cell suspension in a fresh Falcon tube
- Isolate plasmid DNA using the NucleoBond Xtra Maxi Kit (Macherey-Nagel)
- If several electroporations were performed, pool all plasmid samples obtained
- Perform quality controls by Next Generation Sequencing as described in section ***3a)***
- Store the TOPO pool at -20°C

Box 1: When is PCR amplification of the entire oligonucleotide pool appropriate?
In principle it is possible to directly perform amplification of an sgRNA library from the oligo-pool without prior sub-cloning into the TOPO vector. However, often the initial amount of oligonucleotide pool DNA obtained from the vendor is a limiting factor. For example, if 100 ng of DNA are initially provided, but the oligopool contains more than 10 sgRNA libraries, it is not possible to run a PCR

with 10 ng of template for each library. Furthermore, this procedure would not allow to repeat PCR reactions if something went wrong. In contrast, when the oligopool is first sub-cloned into the TOPO vector, the amount of template DNA for any of the sgRNA libraries is virtually infinite. Also, sequencing of the entire pool allows assessing the quality and distribution of the oligopool obtained. A disadvantage of the sub-cloning procedure is the fact that one more PCR, as well as one more cloning step is needed in the entire workflow.

Part 2: Amplification and cloning of specific sgRNA libraries

2a) Amplification of individual sgRNA libraries

- Prepare PCR reactions, using sgRNA library-specific primers for each sgRNA library (PCR 2) to be cloned as follows:

Component	**Amount**
TOPO-pool plasmid DNA	50 pg
sgRNA library-specific forward primer (10 µM stock)	0.75 µl
sgRNA library-specific reverse primer (10 µM stock)	0.75 µl
5X Kapa Hifi Fidelity Buffer	5 µl
dNTP mix (10 mM each)	0.75 µl
Kapa Hifi Polymerase (1 U/µl)	0.5 µl
Water	to total volume of 25 µl

- Perform 1 PCR reaction per 5000 sgRNAs in a library
- Run the PCR as follows:

2 min	98°C	
20 sec	98°C	30 cycles
15 sec	57 - 62°C*	
1 sec	72°C	
2 min	72°C	
forever	4°C	

*set annealing temperature according to the respective primer pair, ideally perform a gradient PCR for each primer pair using your specific setup and thermocycler

- Pool PCR reactions for the same sgRNA library and purify DNA using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel)
- Take an aliquot of the eluted DNA for quality control on a 2% agarose gel

2b) Prepare sgRNA libraries for Gibson cloning

- Amplify an sgRNA library with the vector-specific primers (PCR3) in order to prepare them for subsequent Gibson cloning
- Prepare PCR reactions as follows:

Component	**Amount**
Amplified sgRNA library (from PCR2)	50 pg
H1_f primer or respective PCR3 forward primer (10 µM stock)	0.75 µl
Scaff_r primer or respective PCR3 reverse primer (10 µM stock)	0.75 µl
5X Kapa Hifi Fidelity Buffer	5 µl
dNTP mix (10 mM each)	0.75 µl
Kapa Hifi Polymerase (1 U/µl)	0.5 µl
Water	to total volume of 25 µl

- Perform 1 PCR reaction per 5000 sgRNAs in a library
- Run the PCR as follows:

2 min	98°C	
20 sec	98°C	30 cycles
15 sec	62°C	
1 sec	72°C	
2 min	72°C	
forever	4°C	

- Pool PCR reactions for the same sgRNA library
- If the total volume of all PCR reactions for a given sgRNA library is less than 100 µl bring the volume to 100 µl with water
- Per 100 µl of PCR reaction volume add:
    - 100 µl isopropanol
    - 2 µl 5M NaCl
    - 1 µl GlycoBlue Co-Precipitant
- Vortex samples thoroughly
- Incubate at room temperature for 15 min
- Centrifuge at >16,000 x g for 15 min
- Discard supernatants
- Wash pellets twice with 70% Ethanol
- Air-dry pellets and Resuspend each pellet in 10 µl water

2c) Linearization of the sgRNA expression vector

- Prepare the following reaction:

Component	Amount
sgRNA Expression Vector	5 µg
10X FastDigest Buffer	3 µl
FastDigest BpiI (10 U/µl)	1 µl
Water	to total volume of 30 µl

- Mix by pipetting and incubate at 37°C for 1 h
- Run the entire reaction on a 1% agarose gel
- Excise the band at ~7500 bp
- Purify linearized vector using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel)

2d) Gibson-cloning of sgRNA libraries

- Prepare the following reaction:

Component	Amount
sgRNA Library fragments (from PCR 3)	100 ng
Linearized sgRNA Expression vector	100 ng
2X NEBuilder Hifi Master Mix	5 µl
Water	To total volume of 10 µl

- Perform one reaction per 5000 sgRNAs in a library
- Mix gently by pipetting and briefly spin down the tube
- Incubate at 50°C for 1 h
- Bring volume of each reaction to 100 µl with water
- To each reaction add
  - 100 µl isopropanol
  - 2 µl 5M NaCl
  - 1 µl GlycoBlue Co-Precipitant
- Vortex samples thoroughly
- Incubate at room temperature for 15 min
- Centrifuge at >16,000 x g for 15 min
- Discard supernatants
- Wash pellets twice with 70% Ethanol
- Air-dry pellets and resuspend each pellet in 2 µl water
- Perform electroporation of Endura electrocompetent cells as described in section ***1c)***
  Important: Use 100 µg/ml Ampicillin as selection antibiotic now, don't use Kanamycin

Part 3: Preparing samples for Next Generation Sequencing (NGS)

3a) Preparing oligopools cloned into the TOPO vector for NGS

- Prepare the following two PCR reactions, using different reverse primers:

Component	**Amount**
Cloned TOPO oligopool	50 ng
P5-H1_f primer (10 µM stock)	0.75 µl
P7-TOPO-5p **or** P7-TOPO-3p reverse primer (10 µM stock)	0.75 µl
5X Kapa Hifi Fidelity Buffer	5 µl
dNTP mix (10 mM each)	0.75 µl
Kapa Hifi Polymerase (1 U/µl)	0.5 µl
Water	to total volume of 25 µl

- Run the PCR as follows:

2 min	98°C	
30 sec	98°C	25 cycles
15 sec	62°C	
20 sec	72°C	
2 min	72°C	
forever	4°C	

- Purify DNA according to the requirements of your sequencing facility (Gel purification, Ampure beads etc.)

Box 2: Two PCRs are required to amplify all oligos cloned into the TOPO vector
During the TOPO cloning procedure, the DNA oligos can integrate into the TOPO vector in two different orientations, a process that happens at random, meaning tht roughly 50% of oligos would ligate in the sense and 50% of oligos would ligate in antisense orientation. This is why two PCR need to be performed in order to amplify oligos in both orientations. The PCR is designed in a way that the forward primer binds inside the oligo, while the reverse primers bind the vector backbone, either 5' or 3' of the insertion site.

3b) Preparing sgRNA libraries for NGS

- Prepare the following PCR reaction for each sgRNA library

Component	**Amount**
Cloned sgRNA Library	50 ng
P5-H1_f primer (10 µM stock)	0.75 µl
P7-EF1a_r primer (10 µM stock)	0.75 µl

5X Kapa Hifi Fidelity Buffer	5 µl
dNTP mix (10 mM each)	0.75 µl
Kapa Hifi Polymerase (1 U/µl)	0.5 µl
Water	to total volume of 25 µl

Note: Using 50 ng DNA template amounts to > 5x109 copies of plasmids, which would be > 5000-fold coverage of each plasmid is the library contains one million different constructs. Accordingly, for the vast majority of libraries one PCR reaction is sufficient, otherwise scale-up accordingly.

- Use different combinations of Illumina Sequencing barcodes for different samples in order to allow for multiplexing during sequencing
- Run the PCR as follows:

2 min	98°C	
30 sec	98°C	25 cycles
15 sec	62°C	
20 sec	72°C	
2 min	72°C	
forever	4°C	

- Purify DNA according to the requirements of your sequencing facility (Gel purification, Ampure beads etc.)
- Submit samples to your sequencing facility