THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History®

Protocol for Mini-human library sequencing (Ver 1.0)

Notes:

This library has an identical sequencing design as the Brunello library. Directly adopting Brunello's library sequencing protocol should also work, theoretically.

The protocol is modified from Addgene Brunello library sequencing protocol. (https://www.addgene.org/pooled-library/broadgpp-human-knockout-brunello/)

Materials:

- PCR plates
- NEBNext® Q5® Hot Start HiFi PCR Master Mix (NEB M0543L)
- P5 & P7 primers (listed at the end)
- 70% EtOH
- BD Needle, 27G X1 1/4", Hypodermic, 100/BX (BD Cat No./ID: 305136)
- QIAquick Gel Extraction Kit (Qiagen Cat No./ID: 28706)
- Qubit dsDNA BR Assay Kit (Life Technologies Cat No./ID: Q32850)
- Qubit 4 Fluorometer (Life Technologies Cat No./ID: Q33238)

Procedure

PCR

1. Shear genomic DNA with a 27g needle, or use other methods to reduce the viscosity of template DNA solution. To capture the largest dynamic range of guide abundance change, the total amount of DNA used for PCR ideally should be all DNA; however, ¼ or even less of the total amount is fine. No shearing is needed for plasmid DNA. 300ng plasmid DNA is more than enough if the sequencing of the plasmid is desired.

   Insufficient shearing of DNA will decrease PCR efficiency.

2. Prepare the PCR reaction below

Component	Volume µL
NEBNext® Q5® Hot Start HiFi PCR Master Mix	50
Water	Add up to 100
P5 Primer Mix (100µM)	0.5
P7 Primer (100µM)	0.5
Genomic DNA (µg)	Up to 100µg

   Too much genomic DNA will inhibit PCR reaction, when this happens, reduce the template amount and increase the number of reactions.

3. PCR condition:
    - Initial denaturing at 98°C for 1 min
    - Denaturing at 98°C for 10s, annealing at 64°C for 20s, elongation at 72°C for 30s
    - Final elongation for 2 min, then 4˚C forever

    PCR cycles for each sample should be controlled to the minimal level where the target bands could be seen (usually around 25 cycles) in 2% agarose TAE gel to ensure unbiased PCR amplification. The target band should be ~450bp. Another band at >600 bp will be visible if the template is over-amplified.
4. Gel extraction :

    For PCR reactions that share identical P7 primer, pool them together and load approximately 100 μL for gel extraction with QIAquick Gel Extraction Kit per manufacturer's protocol.
5. Quantify purified DNA concentration with Qubit dsDNA BR Assay Kit
6. Pool all DNA with equal molarity (mass).
7. Proceed for NGS.

Expected reads structure

Part of P5 primer sequence
Guide scaffold
Protospacer sequence (20bp variable)
Sequence from vector

…..GGAAAGGACGAAACACCGTAATTTCTACTCTTGTAGATNNNNNNNNNNNNNNNNNNNN
TAATTTCTACTCTTGTAGATNNNNNNNNNNNNNNNNNNNNTAATTTCTACTCTTGTAGATNN
NNNNNNNNNNNNNNNNNNTAATTTCTACTCTTGTAGATNNNNNNNNNNNNNNNNNNNNTTT
TTTAATTAAGGATCCCCTCGGGGTTAAG……..

PCR primers:

- P5 Primer Sequences

(Mix them together equally before use)
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTGCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTAGCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTCAACTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTTGCACCTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTACGCAACTTGTGGAAAGGACGAAACACCG
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCTGAAGACCCTTGTGGAAAGGACGAAACACCG

- P7 primer

CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTC
CGATCTTCTACTATTCTTTCCCCTGCACTGT

The 8-digit NNNNNNNN should be replaced with the barcode below. Each sample should have a different barcode.

P7 barcode (index) sequences for all designs (NNNNNNNN)					
	Sequence to include in P7 primer, 5'-3'	**Index read (rev comp of sequence)**		**Sequence to include in P7 primer, 5'-3'**	**Index read (rev comp of sequence)**
A01	CGGTTCAA	TTGAACCG	E01	TAACTCAA	TTGAGTTA
A02	GCTGGATT	AATCCAGC	E02	CGTGAGCC	GGCTCACG
A03	TAACTCGG	CCGAGTTA	E03	ATCAGAGG	CCTCTGAT
A04	TAACAGTT	AACTGTTA	E04	TATGGAGG	CCTCCATA
A05	ATACTCAA	TTGAGTAT	E05	GCGTTCAA	TTGAACGC
A06	GCTGAGAA	TTCTCAGC	E06	CGCAAGAA	TTCTTGCG
A07	ATTGGAGG	CCTCCAAT	E07	CGACAGCC	GGCTGTCG
A08	TAGTCTAA	TTAGACTA	E08	CGACTCGG	CCGAGTCG
A09	CGGTGACC	GGTCACCG	E09	TACAAGAA	TTCTTGTA
A10	TACAGAGG	CCTCTGTA	E10	CGCAGATT	AATCTGCG
A11	ATTGTCAA	TTGACAAT	E11	ATTGCTCC	GGAGCAAT

A12	TATGTCTT	AAGACATA	E12	GCACTCGG	CCGAGTGC
B01	ATTGGATT	AATCCAAT	F01	ATGTTCTT	AAGAACAT
B02	ATACTCGG	CCGAGTAT	F02	ATGTCTCC	GGAGACAT
B03	TATGAGAA	TTCTCATA	F03	GCACTCAA	TTGAGTGC
B04	GCACAGTT	AACTGTGC	F04	TAGTAGCC	GGCTACTA
B05	CGTGGATT	AATCCACG	F05	CGTGTCAA	TTGACACG
B06	TAGTAGAA	TTCTACTA	F06	GCGTTCTT	AAGAACGC
B07	GCACGATT	AATCGTGC	F07	GCCAAGCC	GGCTTGGC
B08	CGGTAGCC	GGCTACCG	F08	GCACCTCC	GGAGGTGC
B09	TAGTTCTT	AAGAACTA	F09	GCACCTGG	CCAGGTGC
B10	TACAAGTT	AACTTGTA	F10	GCCAGACC	GGTCTGGC
B11	ATCACTGG	CCAGTGAT	F11	CGCAAGCC	GGCTTGCG
B12	CGCATCAA	TTGATGCG	F12	TACATCAA	TTGATGTA
C01	GCACGACC	GGTCGTGC	G01	GCGTAGCC	GGCTACGC
C02	TACACTCC	GGAGTGTA	G02	CGACAGAA	TTCTGTCG
C03	CGGTCTAA	TTAGACCG	G03	TAGTCTGG	CCAGACTA
C04	ATGTTCGG	CCGAACAT	G04	ATCAAGTT	AACTTGAT
C05	CGTGGACC	GGTCCACG	G05	TAGTAGTT	AACTACTA
C06	ATTGAGCC	GGCTCAAT	G06	ATACTCTT	AAGAGTAT
C07	TAGTTCGG	CCGAACTA	G07	CGGTAGTT	AACTACCG
C08	CGGTGAGG	CCTCACCG	G08	ATACGAGG	CCTCGTAT
C09	CGTGAGTT	AACTCACG	G09	CGCACTGG	CCAGTGCG
C10	ATCAGATT	AATCTGAT	G10	TACAGACC	GGTCTGTA
C11	TAGTGATT	AATCACTA	G11	GCGTGACC	GGTCACGC
C12	CGGTTCGG	CCGAACCG	G12	TATGTCGG	CCGACATA
D01	TATGGACC	GGTCCATA	H01	CGACTCTT	AAGAGTCG
D02	GCCAAGTT	AACTTGGC	H02	GCGTTCGG	CCGAACGC
D03	CGCAGACC	GGTCTGCG	H03	ATACCTAA	TTAGGTAT
D04	CGACCTCC	GGAGGTCG	H04	CGGTGATT	AATCACCG
D05	GCCACTGG	CCAGTGGC	H05	TAACGACC	GGTCGTTA
D06	GCGTAGTT	AACTACGC	H06	ATACAGCC	GGCTGTAT
D07	CGCAAGTT	AACTTGCG	H07	CGACGACC	GGTCGTCG
D08	CGACAGTT	AACTGTCG	H08	ATCACTAA	TTAGTGAT
D09	CGCATCTT	AAGATGCG	H09	CGACCTGG	CCAGGTCG
D10	ATGTTCAA	TTGAACAT	H10	TATGTCAA	TTGACATA
D11	GCGTAGAA	TTCTACGC	H11	TAACCTAA	TTAGGTTA
D12	ATGTAGCC	GGCTACAT	H12	GCCATCTT	AAGATGGC