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MPRA plasmid pool preparation

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protocol .

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Protocol to generate the MPRA plasmid pool.

Gerald Raffl, Boyan Bonev 2021. MPRA plasmid pool preparation. **protocols.io**
https://protocols.io/view/mpra-plasmid-pool-preparation-bxchpit6

protocol ,

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Devices:
Microwave
Blue light illuminator
Centrifuge
Thermal Cycler
Magnet for magnetic bead purifications
Electrophoresis station
Nanodrop
Electroporator

Reagents:
CRE library as single stranded oligos, lyophilized
Nuclease free water (Ambion Invitrogen, Cat. N: AM9937)
NEBNext® Ultra™ II Q5® Master Mix (NEB, Cat. N: M0544)
Eppendorf PCR tubes, 0.2 mL (Eppendorf, Cat. N: 30124359)
DNA LoBind® Tubes, 1.5 mL (Eppendorf, Cat. N: 30108051)
AMPure XP (Beckman Coulter, Cat. N: A63881)
Agarose
TAE Buffer (Tris-acetate-EDTA) (50X) (ThermoFisher Scientific, Cat. N: 10399519)
SYBR™ Safe DNA Gel Stain (ThermoFisher Scientific, Cat. N: S33102)
Quick-Load® Purple 1 kb Plus DNA Ladder (NEB, Cat. N: N0550)
Zymoclean Gel DNA Recovery Kit (Zymo Resarch, Cat. N: D4007)
DNA Clean & Concentrator Kit (Zymo Research, Cat. N: D4014)
Q5® Hot Start High-Fidelity 2X Master Mix (NEB, Cat. N: M0494)
NEBuilder® HiFi DNA Assembly Master Mix (NEB, Cat. N: E2621)
ElectroMAX™ Stbl4™ Competent Cells (ThermoFisher Scientific, 11635018)
Gene Pulser/MicroPulser Electroporation Cuvettes, 0.1 cm gap (BioRad; Cat. N: 1652083)
LB medium, sterile
~30 LB agar plates (10 cm diameter) + 100 µg/mL Carbenicillin
10 LB agar plates (15 cm diameter) + 100 µg/mL Carbenicillin
QIAprep Spin Miniprep Kit (Qiagen, Cat. N: 27106)
KpnI-HF® (NEB, Cat. N: R3142)
SpeI-HF® (NEB, Cat. N: R3133)
EcoRI-HF® (NEB, Cat. N: R3101)
BamHI-HF® (NEB, Cat. N: R3136)
T4 DNA ligase (NEB, Cat. N: M0202)
EndoFree Plasmid Maxi Kit (Qiagen, Cat. N: 12362)

Primers:

name	sequence
pool_amp_F	AGGACCGGATCAACT
pool_amp_R	TCGGTTCACGCAATG
CRS_pMPRA1-GibOH_F	TGCCAGAACATTTCTCTGGCCTAACTGGCCAGGACCGGATCAACT
CRS_GibOH_uniRV_BC12_R	CCGACTAGCTTGGCCGCCGAGGCCGACGCTCTTCCGATCTGNNNNNNNNNNNNNGAATTCATCTGGTACCTCGGTTCACGCAATG
pMPRA1_BB_F	TCGGCGGCCAAGCTAGTC
pMPRA1_BB_R	CAGTTAGGCCAGAGAAATGTTCTGG
RVprimer3(pMPRA1)_F	CTAGCAAAATAGGCTGTCCC
EBV-rev(pMPRA1)_R	GTGGTTTGTCCAAACTCATC

PCR amplification of oligos

1 Design of single stranded oligo library:
5'-AGGACCGGATCAACT**CRE_270bp**CATTGCGTGAACCGA-3'

2 Resuspend the single stranded oligos in nuclease free water to obtain a concentration of 20 ng/µL.

3 To transform the single stranded oligos into double stranded DNA, set up the following PCR reaction:
- 2 µL single stranded oligos
- 5 µL primer pool_amp_F (10µM stock)

- 5 µL primer pool_amp_R (10µM stock)
- 50 µL NEBNext Ultra II Q5 Master Mix
- 38 µL nuclease free water

Split the reaction mix into 4 PCR tubes to avoid PCR jackpotting.

4 Incubate in a PCR cycler with heated lid: 15m
- 30s - 98°C
- 8 cycles (10s - 98°C; 20s - 60°C; 30s - 72°C)
- 5min - 72°C
- hold - 4°C

5 Combine the reaction mix from the 4 PCR tubes in a DNA low binding 1.5 tube. Clean up the PCR product with 1.8X vol. of AMPure XP magnetic beads following the manufacturer's protocol.
Elute the PCR product in 21 µL nuclease free water.

6 ⏸

Determine the DNA concentration by measuring 1 µL on the Nanodrop.

7 Gibson overhangs and a 12bp barcode are added by PCR.
Set up the following PCR reaction:
- variable: 25 ng PCR product from step **go to step #5**
- 5 µL primer CRS_pMPRA1-GibOH_F (10µM stock)
- 5 µL primer CRS_GibOH_uniRV_BC12_R (10µM stock)
- 50 µL NEBNext Ultra II Q5 Master Mix
- to 100 µL: nuclease free water

Split the reaction mix into 4 PCR tubes to avoid PCR jackpotting.

8 Incubate in a PCR cycler with heated lid: 30m
- 30s - 98°C
- 14 cycles (10s - 98°C; 20s - 60°C; 30s - 72°C)
- 5min - 72°C
- hold - 4°C

9 Combine the reaction mix from the 4 PCR tubes and add 20 µL 6X gel loading dye. Perform DNA gel electrophoresis (30 min, 100 V) on a gel made of 1 % agarose in 1X TAE, with 1:30'000 SYBR Safe stain. Cut the band(s) at 399 bp using a blue light illuminator. 40m

10 Extract DNA from the agarose by using the Zymoclean Gel DNA Recovery Kit and following the manufacturer's protocol. Elute in 20 µL nuclease free water. 15m

11 Repurify the product using the DNA Clean & Concentrator Kit (binding buffer ratio 5:1) and following the manufacturer's protocol. Elute in 12 µL nuclease free water. 10m

12 ⏸

Determine the DNA concentration by measuring 1 µL on the Nanodrop.
This is the insert library for the subsequent Gibson assembly. Freeze at -20°C until use.

Amplification of the backbone

13 To obtain more backbone, multiple reactions can be set up in parallel.
Set up the following PCR reaction:
- 1 ng pMPRA1 (addgene #49349)
- 2.5 µL pMPRA1_BB_F (10µM stock)
- 2.5 µL pMPRA1_BB_R (10µM stock)
- 25 µL Q5 Hot Start High-Fidelity 2X Master Mix
- to 50 µL: nuclease free water

- Incubate in a PCR cycler with heated lid: 1h

14
- 30s - 98°C
- 35 cycles (10s - 98°C; 20s - 68°C; 1min - 72°C)
- 5min - 72°C
- hold - 4°C

15 Perform DNA gel electrophoresis and DNA gel extraction (band at 2499bp) as described in steps **go to step #9** - **go to step #10** . Elute in 20 µL nuclease free water.

16 1h

Determine DNA concentration by measuring 1 µL on the Nanodrop.
This is the backbone for the subsequent Gibson assembly. Freeze at -20°C until use.

Gibson assembly

17 Preheat a PCR cycler to 50°C, with lid temperature ≥ 75°C

18 Set up a Gibson assembly reaction in a PCR tube:
- 1 µg backbone from step **go to step #16**
- 2:1 molar ratio (~ 320 ng) insert from step **go to step #12**
- to 50 µL: nuclease free water

19

Add 50 µL NEBuilder HiFi DNA Assembly Master Mix, mix briefly by pipetting up and down and immediately transfer reaction to the PCR cycler at 50°C.
Incubate for 1h at 50°C

20 Purify the product using the DNA Clean & Concentrator Kit (binding buffer ratio 2:1) and following the manufacturer's protocol. Elute in 9 µL nuclease free water.

21 Determine DNA concentration by measuring 1 µL on the Nanodrop.
Ideally, continue with electrotransformation straight away.

Electrotransformation & MPRA pre-pool preparation 30m

22 Prewarm 1 mL SOC medium, ideally to 37°C.
Keep a 1.5 mL Eppendorf tube warm.
Thaw a vial (100 µL) of ElectroMAX Stbl4 Competent Cells on ice.
Precool four electroporation cuvettes on ice.

23 Set electroporator to the following parameters:
- 1.8 kV
- 25 µF
- 200 Ω

24 Mix bacteria with 400 ng of purified Gibson assembly product from step **go to step #21** .
Distribute bacteria to the 4 cuvettes (25 µL each), while keeping them on ice.

25

Electroporate each cuvette with parameters set in step **go to step #23** and immediately add 200 µL warm SOC medium.

26 30m

Combine the 4 reactions in the 1.5 mL Eppendorf tube.
Shake the electrotransformed bacteria for 30min at 37°C and 200 rpm.

27 In the meantime, prewarm 24 LB-agar plates (10 cm diameter) with 100 µg/mL Carbenicillin to 37°C.

28 Vortex bacteria thoroughly and prepare the following 2 conditions:
- condition A: 700 µL bacteria + 800 µL warm LB medium
- condition B: 70 µL bacteria + 1430 µL warm LB medium

29 From **both** conditions, prepare dilutions for counting plates:
- 1:1K dilution (7 µL bacteria + 693 µL warm LB medium)
- 1:10K dilution (70 µL bacteria [1:1K dilution] + 630 µL warm LB medium)

When plating 140 µL, the counting plate has 1'000 times / 10'000 times less colonies than all 10 plates of a certain condition combined.

30 For each condition:
- Plate 140 µL for both counting plates
- Plate 140 µL bacteria mix from step go to step #28 on 10 plates.

31 16h

Label all plates and incubate them at 37°C overnight (min. 16h).

32 Count colonies on counting plates and calculate the number of transformants for each condition. If there is too many colonies, count a quarter of the plate and multiply the count by 4.

e.g.
560 colonies on 1:10'000 dilution & 5200 colonies on 1:1'000 dilution
=> ~ 5.4mio. colonies distributed on 10 plates of this condition

Don't trash the counting plates yet.

33

Depending on the number of CREs in the pool, and the desired number of barcodes per CRE; calculate the required colony number using the following formula:

required colony number = (number of CREs) * (desired number of barcodes per CRE)

Based on the required colony number, choose a condition (undiluted or 1:10 diluted) and scrape the respective number of colonies using cell scrapers and LB medium.

34 Combine the scraped colonies in one tube and vortex to resuspend.
Extract plasmid DNA using the QIAprep Spin Miniprep Kit and following the manufacturer's protocol. Depending on the amount of bacteria, split the mixture into multiple reactions.

35

Determine DNA concentration by measuring 1 µL on the Nanodrop.
Plasmid DNA can be stored at -20°C for months.
This is the MPRA pre-pool which will be used for:
- CRE-Barcode-association sequencing
- Subsequent cloning: insertion of minimal promoter and reporter gene

36 To verify the correct identity of the MPRA pre-pool, perform analytical restriction digest with 250 ng of the MPRA pre-pool.
Set up two separate reactions, following the manufacturer's protocol:
- Linearization with KpnI
- Cut out CRE insert with KpnI and SpeI

37 Perform DNA gel electrophoresis as described in step **go to step #9** .

Linearization: band at ~2850bp

Backbone: band at ~2300bp; Insert: band at 534bp

38

To verify the correct identity of individual clones, pick 16 colonies from a counting plate and inoculate overnight cultures (2 mL LB medium with 100 µg/mL Carbenicillin, shaking at 37°C overnight).

39 Extract plasmid DNA from individual clones using the QIAprep Spin Miniprep Kit and send plasmids for Sanger sequencing using the following primers: RVprimer3(pMPRA1)_F and EBV-rev(pMPRA1)_R

Expected Sanger results, polyN represent the 12bp barcode, introduced in step **go to step #7**

RVprimer3(pMPRA1)_F:
*CTAGCAAAATAGGCTGTCCC*CAGTGCAAGTGCAGGTGCCAGAACATTTCTCTGGCCTAACTGGCCAGGACC
GGATCAACT***CRE_270bp***CATTGCGTGAACCGAGGTACCAGATGAATTCNNNNNNNNNNNNCAGATC
GGAAGAGCGTCGGCCTCGGCGGCCAAGCTAGTCGGGGCGGCCGGCCGCTTCGAGCAGACATGATAAGATA
CATTGATGAGTTTGGACAAACCAC

EBV-rev(pMPRA1)_R:
*GTGGTTTGTCCAAACTCATC*AATGTATCTTATCATGTCTGCTCGAAGCGGCCGGCCGCCCCGACTAGCTTG
GCCGCCGAGGCCGACGCTCTTCCGATCTGNNNNNNNNNNNNNGAATTCATCTGGTACCTCGGTTCACGCAA
TG***CRE_270bp_revcomp***AGTTGATCCGGTCCTGGCCAGTTAGGCCAGAGAAATGTTCTGGCACCTGCA
CTTGCACTGGGGACAGCCTATTTTGCTAG

Restriction digest & ligation of MP_mScarlet-I

40 In this section, the minimal promoter and reporter gene (mScarlet-I) are added to the MPRA pre-pool via restriction cloning.

The plasmid pGR029 (note: also pGR023 and pGR025 - pGR033) contains
- the minimal Promoter (derived from pNL3.1[Nluc/minP], Promega) and
- mScarlet-I (kind gift from the Goetz Lab)

flanked by KpnI and EcoRI restriction sites.

Both pGR029 and the MPRA pre-pool are digested with EcoRI and KpnI. For each reaction, use 5 µg input DNA and 2.5 µL of both enzymes in 50 µL reactions, shaking for 2h at 37°C.

41 Perform DNA gel electrophoresis on the pGR029 digest as described in step **go to step #9** , and cut the relevant band at 779bp.

The MPRA pre-pool does not need to run on the gel, since the 'insert' is just 4 bp long and can be removed by a simple cleanup with the DNA Clean & Concentrator Kit.

42 Extract DNA from the agarose by using the Zymoclean Gel DNA Recovery Kit and following the manufacturer's protocol. Elute in 20 µL nuclease free water.

43 Repurify the gel-extracted product using the DNA Clean & Concentrator Kit (binding buffer ratio 5:1) and following the manufacturer's protocol. Elute in 12 µL nuclease free water. This is the insert for the subsequent ligation reaction.

44 Purify the restriction digest of the MPRA pre-pool using the DNA Clean & Concentrator Kit, and elute in 12 µL nuclease free water. This is the backbone for the subsequent ligation reaction.

45 ⏸

Determine the DNA concentrations by measuring 1 µL on the Nanodrop.
Freeze at -20°C until use.

46 Set up the following ligation reaction:
- 2 µg backbone (digested MPRA pre-pool)
- 2:1 molar ratio of insert (MP_mScarlet-I) => ~ 550 ng
- to 175 µL: nuclease free water
- 20 µL T4 DNA ligase buffer (10X)
- 5 µL T4 DNA ligase

47 Incubate in this order: 30min at 4°C, 4h at 16°C and 30min at RT 5h

48 Purify the ligation product using the DNA Clean & Concentrator Kit (binding buffer ratio 2:1) and following the manufacturer's protocol. Elute in 9 µL nuclease free water.

49 Determine DNA concentration by measuring 1 µL on the Nanodrop.

Electrotransformation & MPRA pool preparation 20h

50 Perform electrotransformation of 100 µL electrocompetent bacteria with 400 ng of purified ligation product as described in go to step #22 - go to step #26 .

51 In the meantime, prewarm 3 normal LB-agar plates (10 cm diameter) and 10 big LB-agar plates (15 cm diameter) with 100 µg/mL Carbenicillin to 37°C.

52 Fill the bacterial culture up to 4 mL with LB medium.

53 Prepare dilutions for counting plates:
- 1:1K: 5 µL bacterial culture + 195 µL LB medium
- 1:10K: 20 µL (1:1K dil.) + 180 µL LB medium
- 1:100K: 20 µL (1:10K dil.) + 180 µL LB medium

54 Plate 140 µL from each counting plate dilution on LB-agar plates (10 cm diameter).
Plate 350 µL of the bacterial culture from step go to step #52 on each of the 10 big LB-agar plates (15 cm diameter).

20h

55 Label all plates and incubate them at 37°C overnight (20h).

56 Count colonies on counting plates and calculate the number of transformants. If there is too many colonies, count a quarter of the plate and multiply the count by 4.

To preserve library complexity, the number of transformants should exceed the desired library complexity from step go to step #33 .

57 Scrape all colonies from the 10 big LB-agar plates using cell scrapers and LB medium. Combine the bacteria and vortex thoroughly to resuspend.

58 Extract plasmid DNA using the EndoFree Plasmid Maxi Kit and following the manufacturer's protocol. In the final step, resuspend the DNA pellet in 1X sterile PBS.

59 ⚠⏸

Determine DNA concentration by measuring 1 µL on the Nanodrop.
This is the final MPRA pool. Freeze in aliquots (!) at -20°C. Repeated freeze-thaw cycles are not recommended!

60 To verify the correct identity of the MPRA pool, perform analytical restriction digest with 250 ng of the MPRA pool. Set up two separate reactions, following the manufacturer's protocol:
- Linearization with BamHI
- BamHI and KpnI

61 Perform DNA gel electrophoresis as described in step go to step #9 .

Linearization: band at ~3.6kbp
BamHI + KpnI: bands at ~2.5kbp and ~1.1kbp