MPRAduo library construction

Introduction

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We developed two different protocols for MPRAduo according to the library type to test. In the reciprocal protocol, we construct two single libraries first and then combine them to make duo libraries for both orientations of elements. In the direct protocol, we construct one single library and then insert another oligo pool to construct one dup library. We used the reciprocal protocol for the benchmarking library and used the direct protocol for the whole-genome RE1 library and non-canonical library.

Materials

- Q5 NEBNext Master Mix (NEB, M0541)
- NEBuilder HiFi DNA Assembly Master Mix (NEB, E2621)
- Q5 High-Fidelity 2X Master Mix (NEB, M0492L)
- AMpure XP (Beckman Coulter, A63881)
- SfiI (NEB, R0123)
- PmeI (NEB, R0560)
- AsiSI (NEB, R0630)
- Exonuclease V (RecBCD) (NEB ,M0345)
- Cut Smart Buffer (NEB, B6004)
- Synthesized oligo (Agilent Technologies or Twist Bioscience)
  - eluted by 50 µL of TE
- pMPRAv3:Δluc:ΔxbaI vector (addgene #109035, A vector)
- pMPRAduo:Δorf vector (P vector)
- pMPRAv3:minP:GFP vector (addgene #109036)
- pMPRAduo:minP:GFP vector
- 10-beta Competent *E. coli* (High Efficiency) (NEB C3019H)
- 10-beta Electrocompetent *E. coli* (NEB C3020K)
- Carbenicillin (Teknova, C8001)
- Plasmid Plus Midi (Qiagen, 12943)
- Plasmid Plus Maxi (Qiagen, 12963)
- Plasmid Plus Mega (Qiagen, 12981)
- Plasmid Plus Giga (Qiagen, 12991)

- SYBR gel stain (Cat# S7563 Life Technologies)
- 384 well plates (Cat# 4483285 Life Technologies)
- Optical Adhesive Film (Cat# 4311971 Life Technologies)
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- Primers
  - 1. MPRA_v3_ampB_F: GCCAGAACATTTCTCTGGCCTAACTGGCCGCTTGACG
  - 2. MPRAduo_libA_10I_R: CCGACTAGCTTGGCCGCCGANNNNNNNNNNTCTAGAGGTTCGTCGACGCGATCGCAGGAGCCGCAGTG
  - 3. MPRAduo_libA_20I_R: CCGACTAGCTTGGCCGCCGANNNNNNNNNNNNNNNNNNNNTCTAGAGGTTCGTCGACGCGATCGCAGGAGCCGCAGTG
  - 4. MPRAduo_libP_F: GCCAGAACATTTCTCTGGCCTAACTGGCCTCGCTTGC
  - 5. MPRAduo_libP_10I_R: GGTACTCACtAGGCCTCGGCNNNNNNNNNNAGCGGCTGCAGCAGGTTTAAACCAGGTCGGCCAGGG
  - 6. MPRAduo_libP_20I_onestp: TCTAGAGGTTCGTCGACGCGATCTCACTAGGCCTCGGCNNNNNNNNNNNNNNNNNNNNAGTCAGCGGCTGCAGCAGGTTTAAACCAGGTCGGCCAGGG
  - 7. MPRAduo_GFP_forA_F: CACTGCGGCTCCTGCGATCTAACTGGCCGGTACCTGAGCTCGCTA
  - 8. MPRAduo_GFP_forA_R: TCTAGAGGTTCGTCGACGCGATATTATTATCATTACTTGTACAGCTCGTCCATGC
  - 9. MPRAduo_GFP_forP_F: CCCTGGCCGACCTGGTTTCTAACTGGCCGGTACCTGAGCTCGCTA
  - 10. MPRAduo_GFP_forP_R: AGTCAGCGGCTGCAGCAGGTTTATTATTATCATTACTTGTACAGCTCGTCCATGC
  - 11. MPRAduo_AintoP_F: CCCAGGCACGACGTTGGTTTACTGGCCGCTTGACG
  - 12. MPRAduo_AintoP_R: AGTCAGCGGCTGCAGCAGGTTTCTAGCTTGGCCGCCGA
  - 13. MPRAduo_PintoA_F: CACTGCGGCTCCTGCGATACTGGCCTCGCTTGC
  - 14. MPRAduo_PintoA_R: TCTAGAGGTTCGTCGACGCGATCTCACTAGGCCTCGGC
  - 19. MPRA_v3_TruSeq_Amp2Sa_F: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTGGCCGCTTGACG
  - 20. libA_seqPCR_N7: CGACGCTCTTCCGATCTNNNNNNNCTAGCTTGGCCGCCGA
  - 21. MPRAduo_TruSeq_2Sa_F2: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGGCCTCGCTTGC
  - 22. libP_seqPCR_N7: CGACGCTCTTCCGATCTNNNNNNNCTCACTAGGCCTCGGC
  - 23. MPRA_Illumina_GFP_F_v2: ACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGCCCTGAGCAAAGACC
  - 24. 4LibMIX_seqPCRr_v2N7: CGACGCTCTTCCGATCTNNNNNNNCATGTCTGCTCGAAGCG
  - 25. TruSeq_Multiplex_Tag_P7: CAAGCAGAAGACGGCATACGAGAT[8bp_index]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
  - 26. TruSeq_Multiplex_Tag_P5: AATGATACGGCGACCACCGAGATCTACAC[8bp index]ACACTCTTTCCCTACACGACGCTCTTCCGATC

Procedure

Shared steps in the reciprocal protocol and the direct protocol

1. Building single ∆orf library

1. Linearize vector

   Mix below and inclubate at 50°C overnight
   Purify using 1x volume of AMpure XP followed by elution using 50 uL of water

Table1

	contents	amount
1	P vector	10 ug
2	Cut Smart Buffer	10 uL
3	SfiI	10 uL
4	water	up to 100 uL

2. PCR E elements

   Mix PCR reaction below for each E element
   Cycle condition: 98°C for 30sec, 20 cycles of (98°C for 10 sec, 60°C for 15 sec, 65°C 45 sec), 72°C for 5 min
   purify using 2x volume of AMpure XP followed by elution using 50 uL of water

Table2

	contents	amount
1	Q5 NEBNext Master Mix	5 uL
2	Primer 4 10 uM	0.5 uL
3	Primer 5 10 uM	0.5 uL
4	gBlock double strand DNA	50 ng
5	water	up to 10 uL

3. Gibson assembly

   Mix below and incubate at 50°C for 40 min
   Transform 1 uL of assembled product to 10-beta Competent E. coli and clone single colony
   Verify insert using Sanger sequencing

Table3

	contents	amount
1	PCR product	1 uL
2	SfiI-digested P-vector	100 ng
3	NEBuilder HiFi DNA Assembly Master Mix	2.5 uL
4	water	up to 5 uL

4. Linearize template vector

Mix below and Incubate at 37°C for 2 hr followed by 65°C 20 min
Dilute digested products to 1 ng/uL

Table4

	contents	amount
1	cloned P-plasmid with insert	500 ng
2	Cut Smart Buffer	1 uL
3	OmeI	0.25 uL
4	water	up to 10 uL

5. Amplify test fragments

Mix PCR reaction below for each E element
Cycle condition: 98°C for 30sec, 6 cycles of (98°C for 10 sec, 60°C for 15 sec, 65°C 45 sec), 72°C for 5 min
Purify PCR product using 2x volume of AMpure XP followed by elution using 20 µL water

Table5

	contents	amount
1	Q5 NEBNext Master Mix	10 uL
2	Primer 4 10 uM	0.5 uL
3	Primer 5 10 uM	0.5 uL
4	Linearized template plasmid	1 uL
5	water	up to 20 uL

6. Pooling and Gibson assembly

Mix PCR products in equalmolar
Mix below and incubate 50°C for 1 hr
Purify assembled products using 1.2x volume of AMpureXP

Table6

	contents	amount
1	Pooled PCR product	600 ng
2	SfiI-digested P-vector	500 ng
3	NEBuilder HiFi DNA Assembly Master Mix	15 uL
4	water	up to 30 uL

7. Transformation, colony harvesting

Transform 1 uL of assmebled product to 10-beta Competent E. coli
Plate to LB plate supplemented with Carbenicillin and incubate overnight
Harvest approximately 100-200 colonies per test sequence by washing plate by PBS
Centrifuge by 4000g for 20 min
Prep plasmid using Plusmid Plus Midi

Reciprocal protocol

2. Building single S ∆orf library

8. Linearize vector

Mix below and incubate at 50°C overnight
Purify using 1x volume of AMpure XP followed by elution using 50 uL of water

Table8

	contents	amount
1	A vector	10 ug
2	Cut Smart Buffer	10 uL
3	SfiI	10 uL
4	water	up to 100 uL

9. Amplify and barcoding oligo library

Mix PCR reaction below
Cycle condition: 98°C for 30sec, 4-6 cycles of (98°C for 10 sec, 60°C for 15 sec, 65°C 45 sec), 72°C for 5 min
Purify PCR product using 2x volume of AMpure XP followed by elution using 50 µL water

Table7

	contents	amount
1	Q5 NEBNext Master Mix	300 uL
2	Primer 1 10 uM	30 uL
3	Primer 3 10 uM	30 uL
4	synthesized oligo pool	6 uL
5	water	up to 600 uL

10. Gibson assembly

Mix below and incubate at 50°C for 60 min
Purify assembled products using 1.2x volume of AMpureXP followed by elution using 50 µL water

Table9

	contents	amount
1	PCR product	2.2 ug
2	SfiI-digested A-vector	2 ug
3	NEBuilder HiFi DNA Assembly Master Mix	50 uL
4	water	up to 100 uL

11. Transformation

Transform assembled product into 10-beta electrocompitent cells with 2kV, 200 ohm, 25 µF
in order to achieve a transformation efficiency equal to 300-times the number of unique oligos sequences
Add 10 mL SOC outgrowth medium, separate to 10 tubes and incubate at 37°C for 1 hr
Independently scaled up in 20 mL of LB supplemented with 100 µg/mL of carbenicillin
Incubate on a shaker at 37°C for 9.5 hr
Prep plasmid library using Plasmid Plus Midi or Maxi

3. Building single orf libraries

12. Linearize single ΔORF vector

Mix below and inclubate at 37°C overnight
Purify using 1x volume of AMpure XP followed by elution using 50 uL of water

Table11

	contents	amount
1	single ∆orf library	10 ug
2	AsiSI or PmeI	10 uL
3	cut smart buffer	10 uL
4	water	up to 100 uL

13. Amplify minP-GFP (1st)

Mix reaction below
Cycle condition: 98°C for 30sec, 20 cycles of (98°C for 10 sec, 60°C for 15 sec, 72°C 45 sec), 72°C for 5 min
add 1 uL of DpnI and incubate at 37°C for 30 min
Purify using 1.5x volume of AMpure XP followed by elution using 50 uL of water

Table10

	contents	amount	for vector A	for vector P
1	Q5 High-Fidelity 2X Master Mix	25 uL		
2	Forward Primer 10 uM	2.5 uL	Primer 7	Primer 9
3	Reverse Primer 10 uM	2.5 uL	Primer 8	Primer 10
4	template plasmid	1 ng		
5	water	up to 50 uL		

14. Amplify minP-GFP (2nd)

Mix reaction below
Cycle condition: 98°C for 30sec, 20 cycles of (98°C for 10 sec, 60°C for 15 sec, 72°C 45 sec), 72°C for 5 min
Purify using 1.5x volume of AMpure XP followed by elution using 50 uL of water

Table12

	contents	amount	for vector A	for vector P
1	Q5 High-Fidelity 2X Master Mix	800 uL		
2	Forward Primer 10 uM	80 uL	Primer 7	Primer 9
3	Reverse Primer 10 uM	80 uL	Primer 8	Primer 10
4	1st PCR product	0.32 uL		
5	water	up to 1600 uL		

15. Gibson assembly

Mix below and incubate at 50°C for 90 min

Purify assembled products using 1.2x volume of AMpureXP followed by elution using 30 µL water

Table13

	contents	amount
1	GFP insert	9 ug
2	AsiSI/PmeI digested library	3 ug
3	NEBuilder HiFi DNA Assembly Master Mix	50 uL
4	water	up to 100 uL

16. RecBCD treatment

Mix below and iucubate at 37°C overnight
Purify with a Monarch PCR & DNA Clean up kit using 12 µL of water for elution

Table14

	contents	amount
1	assembled product	30 uL
2	RecBCD	5 uL
3	AsiSI or PmeI	1 uL
4	NEB Buffer 4	5 uL
5	ATP	1 mM
6	BSA	10 ug
7	water	up to 50 uL

17. Transformation

Transform 3µL of assembled product into 50 µL of 10-beta electrocompitent cells with 2kV, 200 ohm, 25 µF
Add 10 mL SOC outgrowth medium and incubate at 37°C for 1 hr
Scale up in 100 mL of LB supplemented with 100 µg/mL of carbenicillin
Incubate on a shaker at 37°C for 9.5 hr
Prep plasmid library using Plasmid Plus Maxi

4. Assembling duo libraries

18. Amplify oligo-GFP-barcode casette

Mix reaction below
Cycle condition: 98°C for 30sec, 12 cycles of (98°C for 10 sec, 60°C for 15 sec, 65°C 1 min), 72°C for 5 min
Add 2 uL of DpnI and incubate at 37°C for 1 hr
Purify assembled products using 0.6x volume of AMpureXP followed by elution using 100 µL water

Table15

	contents	amount	for vector A	for vector P
1	Q5 High-Fidelity 2X Master Mix	800 uL		
2	Forward Primer 10 uM	80 uL	Primer 11	Primer 13
3	Reverse Primer 10 uM	80 uL	Primer 12	Primer 14
4	single ORF plasmid library	32 ng		
5	water	up to 1600 uL		

19. Gibson assembly

Mix below and incubate at 50°C for 90 min. Use linearized vector from step 12.
Purify assembled products using 1.2x volume of AMpureXP followed by elution using 75 µL water

Table16

	contents	amount
1	Amplified inserts	4 ug
2	AsiSI/PmeI digested single ΔORF library	2 ug
3	NEBuilder HiFi DNA Assembly Master Mix	100 uL
4	water	up to 200 uL

20. RecBCD treatment

Mix below and iucubate at 37°C overnight
Purify with a Monarch PCR & DNA Clean up kit using 12 µL of water for elution

Table17

	contents	amount
1	assembled product	75 uL
2	RecBCD	5 uL
3	AsiSI	1 uL
4	PmeI	1 uL
5	NEB Buffer 4	10 uL
6	ATP	1 mM
7	BSA	10 ug
8	water	up to 100 uL

21. Transformation

Transform 3µL of assembled product into 50 µL of 10-beta electrocompitent cells with 2kV, 200 ohm, 25 µF
Add 10 mL SOC outgrowth medium, separate to 5 tubes and incubate at 37°C for 1 hr
Scale up in 5 x 100 mL of LB supplemented with 100 µg/mL of carbenicillin
Incubate on a shaker at 30°C for 16 hr
Prep plasmid library using Plasmid Plus Mega

Direct protocol

6. Assembling a duo ΔORF library

Here we describe the protocol to insert P vector into A vector. When inserting P vector into A vector, change vector plasmids and restriction enzymes appropriately.

22. Linearize single ΔORF vector

Mix below and inclubate at 37°C overnight
Purify using 1x volume of AMpure XP followed by elution using 50 uL of water

Table20

	contents	amount
1	single Δorf library (plasmid A)	10 ug
2	AsiSI	10 uL
3	cut smart buffer	10 uL
4	water	up to 100 uL

23. Amplify and barcoding oligo library

Mix PCR reaction below
Cycle condition: 98°C for 30sec, 4-6 cycles of (98°C for 10 sec, 60°C for 15 sec, 65°C 45 sec), 72°C for 5 min
Purify PCR product using 2x volume of AMpure XP followed by elution using 50 µL water

Table21

	contents	amount
1	Q5 NEBNext Master Mix	300 uL
2	Primer 2 10 uM	30 uL
3	Primer 6 10 uM	30 uL
4	synthesized oligo pool	6 uL
5	water	up to 600 uL

24. Gibson assembly

Mix below and incubate at 50°C for 60 min
Purify assembled products using a Monarch PCR & DNA Clean up kit using 12 µL of water for elution

Table18

	contents	amount
1	PCR product (step 23)	220 ng
2	linearized single ΔORF vector (step 22)	2 ug
3	NEBuilder HiFi DNA Assembly Master Mix	100 uL
4	water	up to 200 uL

25. Transformation

Transform assembled product into 10-beta electrocompitent cells with 2kV, 200 ohm, 25 µF
in order to achieve a transformation efficiency equal to 200-300 times the number of unique oligos sequences
Add 10 mL SOC outgrowth medium, separate to 10 tubes and incubate at 37°C for 1 hr
Independently scaled up in 20 mL of LB supplemented with 100 µg/mL of carbenicillin
Incubate on a shaker at 37°C for 9.5 hr
Prep plasmid library using Plasmid Plus Maxi

26. Amplify minP-GFP

Follow step 13-14

27. Linearize duo ΔORF vector

Mix below and inclubate at 37°C overnight
Purify using 1x volume of AMpure XP followed by elution using 50 uL of water

Table19

	contents	amount
1	duo Δorf library	10 ug
2	PmeI	10 uL
3	cut smart buffer	10 uL
4	water	up to 100 uL

28. Gibson assembly

Mix below and incubate at 50°C for 90 min. Use linearized vector from step 12.
Purify assembled products using 1.2x volume of AMpureXP followed by elution using 75 µL water

Table22

	contents	amount
1	Amplified GFP inserts	4 ug
2	PmeI digested duo ΔORF library	2 ug
3	NEBuilder HiFi DNA Assembly Master Mix	100 uL
4	water	up to 200 uL

29. RecBCD treatment

Mix below and iucubate at 37°C overnight
Purify with a Monarch PCR & DNA Clean up kit using 12 µL of water for elution

Table23

	contents	amount
1	assembled product	75 uL
2	RecBCD	5 uL
3	AsiSI	1 uL
4	PmeI	1 uL
5	NEB Buffer 4	10 uL
6	ATP	1 mM
7	BSA	10 ug
8	water	up to 100 uL

30. Transformation

Transform 3µL of assembled product into 50 µL of 10-beta electrocompitent cells with 2kV, 200 ohm, 25 µF
Add 12 mL SOC outgrowth medium, separate to 6 tubes and incubate at 37°C for 1 hr
Scale up in 6 x 500 mL of LB supplemented with 100 µg/mL of carbenicillin
Incubate on a shaker at 30°C for 16 hr
Prep plasmid library using Plasmid Plus Giga

Preparation of Illumina sequencing libraries for oligo-barcode matching

31. Amplify oligo-barcode fragment from single ΔORF vector

Mix reaction below
Cycle condition: 98°C for 30sec, 5 cycles of (98°C for 10 sec, 62°C for 15 sec, 72°C 1 min), 72°C for 2 min
Purify assembled products using 1x volume of AMpureXP followed by elution using 30 µL EB

Table24

	contents	amount	for A vector	for P vector
1	Q5 High-Fidelity 2X Master Mix	100 uL		
2	Forward Primer 10 uM	10 uL	Primer 19	Primer 21
3	Reverse Primer 10 uM	10 uL	Primer 20	Primer 22
4	single ORF plasmid library	400 ng		
5	water	up to 200 uL		

32. Amplify oligo-barcode fragment with Illumina index

    Mix reaction below
    Cycle condition: 98°C for 30sec, 6 cycles of (98°C for 10 sec, 62°C for 15 sec, 72°C 1 min), 72°C for 2 min
    Purify assembled products using 1x volume of AMpureXP followed by elution using 30 µL EB

Table25

	contents	amount	for both vector
1	Q5 High-Fidelity 2X Master Mix	50 uL	
2	Forward Primer 10 uM	5 uL	Primer 25
3	Reverse Primer 10 uM	5 uL	Primer 26
4	PCR product from step 31	20 uL	
5	water	up to 100 uL	

33. Sequence using 2x250 bp chemistry

Preparation of Illumina sequencing libraries for Tag-seq

34. Prepare cDNA following **MPRAduo library transfection** protocol.

35. Serial dilute plasmid library from 1000pg to 1fg using 10 fold dilutions.

    Use 1000 uL per dilution
    Use EB + 0.01% SDS for dilutions.

36. qPCR Setup for normalization and cycle determination

    Mix reaction below
    Cycle condition: 98°C for 30sec, 40 cycles of (98°C for 10 sec, 62°C for 15 sec, 72°C 1 min), 72°C for 2 min, Melt Curve Analysis

Table26

	contents	amount	for both vector
1	Q5 High-Fidelity 2X Master Mix	5 uL	
2	Forward Primer 10 uM	0.5 uL	Primer 23
3	Reverse Primer 10 uM	0.5 uL	Primer 24
4	cDNA/diluted plasmid	1 uL	
5	SYBR Gel Stain (1/6000 diluted)	1 uL	
6	water	up to 10 uL	

37. Analyze the result of qPCR

Find the CT (cycle) when the amplification curve for the cDNA qPCR just begins to take off and subtract one cycle.
Use this number of cycles in the first illumina prep PCR.

38. 1st PCR

Mix reaction below
Cycle condition: 98°C for 30sec, **N** cycles of (98°C for 10 sec, 62°C for 15 sec, 72°C 1 min), 72°C for 2 min
Purify assembled products using 1x volume of AMpureXP followed by elution using 30 µL EB

Table27

	contents	amount	for both vector
1	Q5 High-Fidelity 2X Master Mix	50 uL	
2	Forward Primer 10 uM	5 uL	Primer 23
3	Reverse Primer 10 uM	5 uL	Primer 24
4	cDNA/diluted plasmid	10 uL	
5	water	up to 100 uL	

39. 2nd PCR

Mix reaction below
Cycle condition: 98°C for 30sec, 6 cycles of (98°C for 10 sec, 62°C for 15 sec, 72°C 1 min), 72°C for 2 min
Purify assembled products using 1x volume of AMpureXP followed by elution using 30 µL EB

Table28

	contents	amount	for both vector
1	Q5 High-Fidelity 2X Master Mix	50 uL	
2	Forward Primer 10 uM	5 uL	Primer 25
3	Reverse Primer 10 uM	5 uL	Primer 26
4	1st PCR product from step38	20 uL	
5	water	up to 100 uL	

40. Sequence using 2x150 bp or 1x150 bp chemistry