MPRAv3 Library Generation Protocol

Introduction

Protocol for the construction of massively parallel reporter assays from an oligo library.

Materials

Primers/Oligos

- #82 - MPRA_v3_F: GCCAGAACATTTCTCTGGCCTAACTGGCCGCTTGACG
- #202 - MPRA_v3_20I_R: CCGACTAGCTTGGCCGCCGACGCTCTTCCGATCT(N1:25252525)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)(N1)TCTAGAGGTTCGTCGACGCGATCGCAGGAGCCGCAGTG
- #200 - MPRA_v3_GFP_Fusion_v2_F: CACTGCGGCTCCTGCGATCTAACTGGCCGGTACCTGAGCTCGCTA
- #201 - MPRA_v3_GFP_Fusion_v2_R: TCTAGAGGTTCGTCGACGCGATTATTATCATTACTTGTACAGCTCGTCCATGC
- #197 - MPRA_v3_TruSeq_Amp2Sa_F: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTGGCCGCTTGACG
- #757 - Ilmn_P5_1stPCR: ACACTCTTTCCCTACACGACGCTCTTCCGATC

Vectors

- pMPRAv3:Δluc:ΔxbaI (Addgene #109035)
- pMPRAv3:minP-GFP (Addgene #109036)

Other

- SfiI restriction enzyme (NEB)
- AsiSI restriction enzyme (NEB)
- DpnI
- NEB q5 Master Mix
- NEB 2x Gibson Master Mix
- 10-beta e.coli (NEB or Lucigen)
- Qiagen plasmid plus maxi/giga kits
- Qiagen plasmid plus maxi/giga kits
- RecBCD (NEB, M0345)
- NEB 10x Buffer 4

Procedure

PCR LIBRARY

1. PCR amplify library after resuspending in 50 uL
2. Perform a test PCR amplification using 1x reaction with 25 cycles and run unpurified product on fresh 1.2% Flash Gel to make sure the band is crisp. Then perform a full scale up of 12 reactions.

Component	Vol (uL)	12x
Q5 2X MM (NEBNEXT Hot-Start) Ultra II	25	300
Primer MPRA_v3_F (#82) (10 uM)	2.5	30
Primer MPRA_v3_20I_R (#202) (10 uM)	2.5	30
DNA LIBRARY	0.5	6
dH20	19.5	234
TOTAL	50	

3. Cycle conditions: 98C for 30s; 6x {98C for 10s; 60C for 15s; 65C for 45s}; 72C for 5m; 4C
4. SPRI library at 0.8x, wash with 80% EtOH (Higher SPRI concentrations can be used depending on the project and oligos)
   PCR product = Oligo (230bp) + F primer (22bp addition) + R primer (77bp addition) = 329bp total library

5. Run library pool after spri on 2.2% flash gel to ensure amplification and lack of smearing
6. Quantify on Nanodrop

MPRA Vector Assembly - mpra∆orf library

7. Digest backbone

Component	Target ug	Vol (uL)
10x CutSmart Buffer		10
SfiI		10
pMPRAv3:∆luc:∆xbaI	10ug	
dH20		80.00
TOTAL		100.00

8. Incubate at 50C overnight
9. SPRI at 1.5x to cleanup
10. Run 1.2% Lonza gel of cut and uncut vector to ensure proper digestion
11. Setup gibson assembly reaction

Component	Target ug	1x
2x NEB HiFI Assembly MM		50.00
DNA oligo pool	2.2ug	
SfiI digested - pMPRAv3:∆luc:∆xbaI	2ug	
dH20		50.00
TOTAL		100.00

12. Incubate at 50C for 1 hr
13. SPRI purification at 1.2X and elute in 20 µL. Store in Lo-bind tubes. (need 100-150ng/ul to get 3x106 CFU count)
14. Test transformation to dial in CFU count: 1ul (~100ng/ul) Gibson and 50ul 10 beta electrocompetent cells electroporation (2kV, 200 ohm, 25 µF), add 950 SOC. Incubate for 1 hour at 37C while rotating. Dilute 1:100, 1:1000, 1:10000, 1:100000. Calculate CFUs. Increase or decrease the amount of original 1ml to use for 1 hour culture incubation according to desired CFU count.
15. Transform 1ul of ligated vector into 50 µL 10-beta e.coli (dependent on test transformation) by electroporation (2kV, 200 ohm, 25 µF). Setup 1x 50ul electroporations add 950 ul SOC to each. Add the appropriate amount of 1ml culture to obtain desired CFU count.
    The typical MPRA library uses ~200 barcodes per unique oligo sequence tested.
    (CFU = 200 x # of oligos)
16. Split into ten 1 mL aliquots of SOC, rotate tubes for 1 hour at 37°C. Independently expand in 20 mL of LB supplemented with 100 µg/mL of carbenicillin in shaker at 37°C for 6.5 hours.
17. Serial dilute and create CFU counting plates from initial SOC split to determine library complexity/size
18. Pool aliquots to reach desired CFU complexity and purify with Qiagen Plasmid Plus maxi/midi kit.
19. Pick 8-16 colonies for colony PCR and Sanger sequencing with primers #1/#2 to confirm insert.

Illumina Sequencing for oligo-tag association & complexity check

20. PCR1: Capture oligo + BC combination from pMPRAv3:∆orf plasmid

Component	Vol (uL)
Q5 2X MM (NEBNEXT Hot-Start) Ultra II	50
TruSeq_Universal_Adapter (10um) #757	5.00

MPRA_v3_TruSeq_Amp2Sa_F (10um) #197	5.00
pMPRA:Δorf Plasmid (200ng)	4.00
dH20	36.00
TOTAL	100.00

21. Cycle conditions: 98C for 30s; 5x {98C for 20s; 62C for 15s; 72C for 30s}; 72C for 2m; 4C
22. Spri at 1X and elute in 30 of EB
23. PCR2: Attach index/final illumina adapters

Component	Vol (uL)
Q5 2X MM (NEBNEXT Hot-Start) Ultra II	50
P5 index primer #___(10um)	5.00
P7 index primer #___(10um)	5.00
Eluted PCR1 product	**20.00**
dH20	20.00
TOTAL	100.00

24. Cycle conditions: 98C for 30s; 5x {98C for 10s; 62C for 15s; 72C for 30s}; 72C for 2m; 4C
25. Spri at 1X and elute in 30 of EB
26. Tapestation to quantify and pool based on molarity
27. Sequence on Illumina using 2x150+bp.

MPRA Vector Assembly - mpraΔgfp library

28. Linearize library with AsiSI

Component	Target ug	Vol (uL)
10x CutSmart Buffer		40.00
AsiSI	100 units	10.00
pMPRAv3:Δluc:ΔxbaI w/ library	10ug	
dH20		350.00
TOTAL		400.00

29. Incubate at 37C overnight
30. Purify in NEB Monarch Columns (5ug capacity). Use the appropriate number of columns. Elute in 30ul EB one column at a time using elution from column one to elute column two.
31. Generate GFP amplicon using PCR (GFP:amp1)

Component	1x
Q5 2X MM (NEBNEXT Hot-Start)	25.00
primer #200	2.50
primer #201	2.50
pMPRAv3:minP-GFP (0.1 ng/uL)	1.00
dH20	19.00
TOTAL	50.00

32. Cycle conditions: 98C for 30s; 20x {98C for 10s; 60C for 15s; 72C for 45s}; 72C for 5m; 4C
33. Add Dpn1 digestion to PCR mixture and incubate for 30 min at 37C
34. Double SPRI with .5x to remove bigger band, then 1.5x spri, elute in 40ul
35. 2nd PCR of GFP amplicon (GFP:amp2)

Component	1x	32x
Q5 2X MM (NEBNEXT Hot-Start)	25.00	800
primer #200	2.50	80
primer #201	2.50	80
GFP:amp1 PCR diluted 1:100	0.40	12.8
dH20	19.60	627.2
TOTAL	50.00	

36. Cycle conditions: 98C for 30s; 20x {98C for 10s; 60C for 15s; 72C for 45s}; 72C for 5m; 4C
37. Pool reactions, 1.5x SPRI, serial elute in 100 ul
38. Purify in Qiagen Qiaquick (10ug capacity). Use 4 columns. Serial elute columns with 100 ul EB.
39. Insert GFP orf using Gibson Assembly

Component	Target ug	1x
2x Gibson Master Mix		125.00
GFP amplicon	5.28 ug	
digested pMPRAv3:Δluc:ΔxbaI w/ library	1.6 ug	
dH20		125.00
TOTAL		250.00

40. Incubate at 50C for 90 min
41. 1.5x SPRI purify elute in 40 ul
42. Run Gibson on 1.2% Lonza Flash gel to ensure there is a band above 4kb
43. Re-digest to remove uncut vector

Component	Target ug	1x
10x Buffer 4	1x	10.00
AsiSI	50 units	2.50
RecBCD	5 units	0.50
BSA	10ug	1.00
ATP	1mM	
pMPRAv3:Δluc:ΔxbaI w/ library	all gibson product	
dH20		86.00
TOTAL		100.00

44. Incubate at 37C overnight.
45. 1.5x SPRI purify elute in 40 ul
46. At this stage you can perform a test transformation to evaluate the efficiency of the GFP insertion or proceed directly to the full scale library prep. Test transformation to dial in CFU count: 2ul (~100ng/ul) Gibson and 50ul 10 beta electrocompetent cells electroporation (2kV, 200 ohm, 25 µF), add 950 SOC. Incubate for 1 hour at 37C while rotating. Dilute 1:100, 1:1000, 1:10000, 1:100000. Calculate CFUs. Increase or decrease the amount of original 1ml to use for 1 hour culture incubation according to desired CFU count.

Full Scale MPRA Plasmid Preparation

47. Electroporate 10 ul (2kV, 200 ohm, 25 µF) into 220 µL 10-beta cells or use amounts of Gibson and cells discovered in test transformation
48. Immediately split electroporated bacteria across 6 tubes and recover each in 2 mL of SOC for 1 hour at 37°C
49. After recovery each tube is independently added to 500 mL of TB with 100 µg/mL of carbenicillin and grown for 16 hours at 30°C prior (3 L total)

Create CFU counting plates for 4 of the 500 mL cultures to evaluate transformation efficiency

50. After 16 hours of growth spin cultures and process pellet using the Qiagen Plasmid Plus Giga protocol
51. Pick 8-16 colonies from CFU plate for colony PCR and Sanger sequencing with primers #1/#2 to confirm insert.
52. Qiagen Plasmid Plus Giga Prep to extract plasmid from bacteria. Do not exceed 7.5g bacterial cell pellet weight per column. Also, do not exceed -300mBar vacuum pressure on any of the vacuum steps or you will get bacterial genomic DNA contamination in your library.
53. Run the purified product on a gel to confirm plasmid quality. If genomic or RNA contamination is observed, repurify the plasmid a second time using a Qiagen Plasmid Plus Giga column.

MPRA RNA Extraction - Maxi Scale

Introduction

Standard MPRA protocol for RNA Extraction and GFP Capture from $1x10^8$ - $5x10^8$ cells. If greater than $5x10^8$ cells need to be processed split cells across multiple RNeasy columns and pool prior to GFP capture.

Materials

Primers/Oligos

- 33 uM Primer #120: CCTCGATGTTGTGGCGGGTCTTGAAGTTCACCTTG/3BioTEG/
- 33 uM Primer #123: CCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCC/3BioTEG/
- 33 uM Primer #126: CGCCGTAGGTGAAGGTGGTCACGAGGGTGGGCCAG/3BioTEG/

Other

- Qiagen RNeasy Maxi Kit (Qiagen #)
- Molecular Biology Grade Ethanol (Fisher #BP2818500)
- RNase-free Water (Life Tech #10977023 or AM9937)
- SUPERase-In (Life Tech #AM2696)
- TURBO DNase (Life Tech #AM2239)
- 10% SDS Solution (Life Tech #15553027)
- 0.5M EDTA (Life Tech #AM9260G)
- 20X SSC (Life Tech#15557044)
- Deionized Formamide (VWR #EM-4650)
- GFP 3 Probe Mix (100 uM total)
- Dynabeads MyOne Streptavidin C1 (Life Tech #65002)
- Bead Buffer WashA
  - 0.1 M NaOH
  - 0.05 M NaCl
- Bead Buffer WashB
  - 0.1 M NaCl
- SuperScript III First-Strand Synthesis SuperMix (Life Tech #18080400)
- First Strand Gene Specific Primer
- 20 uM Primer #19: CCGACTAGCTTGGCCGC
- Agencourt RNAClean XP (Beckman #A63987)
- Agencourt AMPure XP (Beckman #A63881)

Procedure

RNA Extraction

Notes

Add 4 volumes of EtOH (96-100%) to Buffer RPE before using.
Skip steps 1-3 if RLT/DTT and lysing was performed prior to freezing.

1. Remove frozen cell pellet from -80C and immediately add 7.5/15 mL Qiagen RLT Buffer and 150/300 µL 2M DTT (<$2x10^8$/>$2x10^8$ cells)
2. Dip tubes in 37 degree bath for 30 sec to defrost the pellet
3. Using a 10 mL syringe and 21 gauge needle aggressively draw up and dispense the cell lysis 5 times.
4. Add 7.5 (15 for >$2x10^8$ cells) mL of 70% EtOH to the cell lysis
5. Transfer 15 mL of lysis/EtOH mixture to an RNeasy Maxi column and spin at 4000 x g for 5 min and discard flow through. (For >$2x10^8$ cells add remaining lysate to the RNeasy Midi column and spin at 4000 x g for 5 min and discard flow through.)
6. Add 7.5 mL of Qiagen RW1 buffer to the column and spin at 4000 x g for 5 min
7. Mix 30 µl of Qiagen DNase and 210 µl of Qiagen RDD buffer. Flick gently and centrifuge to collect the liquid. (x5 for five replicates)

8. Add DNase mixture to each column dropwise evenly over the entire filter.
9. Incubate for 15 minutes.
10. Add 7.5 mL of Qiagen RW1 buffer to the column and spin at 4000 x g for 5 min, discard flow through
11. Add 10 mL of Qiagen RPE buffer to the column and spin at 4000 x g for 2 min, discard flow through
12. Add 10 mL of Qiagen RPE buffer to the column and spin at 4000 x g for 2 min, discard flow through
13. Spin empty column at 4000 x g for 10 min
14. Add 750 µl of water to the column, let sit for 1 min and spin at 4000 x g for 2 min
15. Add 900 µl of water to the column, let sit for 1 min and spin at 4000 x g for 5 min Combine elutions, add 5 µl of SUPERase•In
16. Optional: Use 1 µL for rtPCR to check for GFP transcripts

Pre-capture DNase Treatment

17. Setup DNase reaction

Component	Vol (uL)
Total RNA	1475
Turbo DNase 10x Buffer	165
Turbo DNase	10

18. Incubate for 1 hour at 37C
19. Post incubation add the following:
    15 uL of 10 % SDS
    150 uL of 0.5M EDTA
20. Incubate for 5 minutes at 70C then immediately place on ice

GFP Transcript Pulldown

21. Combine the following in a 5 mL Low-Bind tube:

Component	Vol (uL)
DNase treated RNA	1800
SSC 20X	600
Formamide	1200
GFP 3 Probe Mix - 100 uM	2

22. Mix tube with vortex
23. Incubate for 2.5 hours at 65C
24. Prepare capture beads during Incubation:
    - In a 1.5 mL Low-Bind Tube wash 400 µl (multiply by the # of replicates) of Life Technologies C1 beads 2x wash with WashA Bufffer (0.1 M NaOH, 0.05 M NaCl) with 5 minute incubations
    - 1x wash with WashB Buffer (0.1 M NaCl)
    - Elute beads with 500 µl (multiply by the # of replicates) of 20x SSC
    - Add 500 µl of RNase clean C1 beads in 20x SSC to samples in 5 mL tube
25. Mix on rotator at room temperature for 15 minutes
26. Place 5 mL tube on a magnet and remove supernatant
27. Wash with 500 µl 1x SSC and transfer to a 1.5 mL tube
28. Place 1.5 mL tube on a magnet and remove supernatant

29. Wash with 500 µl 0.1x SSC
30. Wash with 500 µl 0.1x SSC
31. Add 50 µl of H2O
32. Add 1 ul of SUPERase•In to the ~50 ul elution and proceed to the DNase treatment. Leave the beads in the reaction mix.

Post-capture DNase Treatment

33. Combine the following:

Component	Vol (uL)
Total RNA + water	53.5
Turbo DNase 10x Buffer	5.5
Turbo DNase	1

34. Incubate for O/N hour at 37C
35. Post incubation add the following:
    1 uL of 10 % SDS
36. Purify with 2x RNA Ampure XP
37. Elute RNA in 37 uL of H_2O
38. Add 1 ul of SUPERase•In

GFP Transcript Reverse Transcriptase

39. Combine the following for primer annealing

Component	Vol (uL)
DNase treated GFP mRNA	33
Annealing Buffer	5
Primer #19 - 20 uM	2

40. Incubate for 5 minutes at 65C to a 4C hold. Keep on ice after incubation
41. Add the following to the reaction on ice:
    50 uL of 2X First-Strand Reaction Mix
    10 uL of Enzyme Mix
42. Incubate for 80 minutes at 47˚, followed by 5 minutes at 85˚
43. Cleanup with 2x Ampure XP
44. Elute cDNA in 30 ul EB

Tag-seq Library Preparation for Illumina Sequencing

Introduction

Generation of Illumina sequencing libraries from GFP cDNA (non-UMI)

Materials

Primers/Oligos

- #801 - MPRA_Illumina_GFP_F_v2: ACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGCCCTGAGCAAAGA*C*C
- #802 - Ilmn_P5_1stPCR_v2: ACTCTTTCCCTACACGACGCTCTTCCGAT*C*T
- Illumina P5 Index Primers: AATGATACGGCGACCACCGAGATCTACAC[8-10bp Index]ACACTCTTTCCCTACACGAC
- Illumina P7 Index Primers: CAAGCAGAAGACGGCATACGAGAT[8-10bp Index]GTGACTGGAGTTCAGACGTGTGC

Other

- cDNA generated from GFP RNA (RNA concentration is usually very low, less than 1ng/ul) from harvested transfected cells. Need at least 4 replicates.
- Final MPRA plasmid library
- GFP RNA from harvested transfected cells
- Applied Biosystems Viia7
- 384 well plates (Cat# 4483285 Life Technologies)
- Optical Adhesive Film (Cat# 4311971 Life Technologies)
- SYBR gel stain (Cat# S7563 Life Technologies)
- Q5 Ultra Next (Cat# M0544S NEB)

Procedure

qPCR Setup for normalization and cycle determination

1. Serial dilute plasmid library from 1000pg to 1fg using 10 fold dilutions.
   Use a minimum volume of 500 uL per dilution
   Use EB + 0.01% SDS for dilutions.
2. Run plasmid samples, post-capture/DNase'd RNA, NTC and cDNA samples in duplicate

Component	Vol (uL)
Q5 NEBNEXT Ultra II 2X MM	5
Primers 781 (10 uM)	0.5
Primers 782 (10 uM)	0.5
Sybrgreen I (1:10,000)	1.66
cDNA	1
dH20	1.34
Total	10

3. Cycle conditions: 98C for 30s; 40x {98C for 10s; 62C for 15s; 72C for 30s}; 72C for 2m; 4C; Melt Curve Analysis
4. Analyze the results of qPCR
5. 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.
6.

Starting Quantification	1st PCR Cycle #
750 pg	9
400 pg	10
200 pg	11
100 pg	12

Data derived from historical averages in the Tewhey lab. Starting quantification equals the total mass of cDNA used as input for PCR 1.

7. Illumina PCR 1 Setup

Component	Vol (uL)
Q5 NEBNEXT Ultra II 2X MM	25
Primers 782 (10 uM)	2.5
Primers 781 (10 uM)	2.5
cDNA	
dH20	
Total	50

8. Cycle conditions: 98C for 20s; cycles determined by qPCR {98C for 10s; 62C for 15s; 72C for 30s}; 72C for 2m; 4C
9. Spri at 1X and elute in 30 of EB
10. Second PCR 2 setup

Component	Vol (uL)
Q5 NEBNEXT Ultra II 2X MM	25
ILMN P5 Index Primer (10 uM)	2.5
ILMN P7 Index Primer (10 uM)	2.5
Eluted PCR product	20
dH20	0
Total	50

11. Cycle conditions: 98C for 20s; 6x {98C for 10s; 62C for 15s; 72C for 30s}; 72C for 2m; 4C
12. Spri at 1X and elute in 30 of EB
13. Qubit/Tapestation to quantify and confirm size if running multiple samples.
14. Sequence on Illumina with a 1x21bp read. Cluster at 80-90% max density with a 5-10% PhiX spike