Nov 30, 2021

MPRA library preparation

Gerald Raffl[1], Boyan Bonev[1]
[1]Helmholtz Pioneer Campus

1

protocol .

Boyan Bonev
Helmholtz Pioneer Campus

Protocol to generate both DNA and RNA MPRA libraries.

Gerald Raffl, Boyan Bonev 2021. MPRA library preparation. **protocols.io**
https://protocols.io/view/mpra-library-preparation-bxdtpi6n

protocol ,

Aug 16, 2021

Nov 30, 2021

52371

Devices:
Centrifuge
2 ThermoMixers (Eppendorf)
Thermal Cycler
Magnet for magnetic bead purifications
Qubit fluorometer (ThermoFisher Scientific)
Real-Time Thermal Cycler for qPCR
Agilent 2100 Bioananlyzer (Agilent)

Reagents:
Nuclease free water (Ambion Invitrogen, Cat. N: AM9937)
2X digestion buffer (Zymo Research, Cat. N: D3050-1)
Proteinase K (lyophilized) & Storage Buffer (Zymo Research, Cat. N: D3001-2)
Quick-DNA/RNA Microprep Plus Kit (Zymo Research, Cat. N: D7005)
TURBO DNA-free™ Kit (ThermoFisher Scientific, Cat. N: AM1907)
Qubit™ Assay Tubes (ThermoFisher Scientific, Cat. N: Q32856)
Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Cat. N: Q32854)
Qubit™ RNA HS Assay Kit (ThermoFisher Scientific, Cat. N: Q32852)
RNA 6000 Pico Kit (Agilent, Cat. N: 5067-1513)
Maxima H Minus Reverse Transcriptase (200 U/µL) (ThermoFisher Scientific, Cat. N: EP0753)
Oligo(dT)18 Primer (ThermoFisher Scientific, Cat. N: SO132)
dNTP Solution Mix (NEB, Cat. N: N0447)
RNasin® Plus Ribonuclease Inhibitor (Promega, Cat. N: N2611)
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)

Luna® Universal qPCR Master Mix (NEB, Cat. N: M3003)
High Sensitivity DNA Kit (Agilent, Cat. N: 5067-4626)

Primers:

name	sequence
RV_univ_MPRA	CGACGCTCTTCCGATCT
FWD_mScar_Tn7_10UMI_3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNTCGACCGCAAGTTGGAC
FWD_CRS_Tn7	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCAGGACCGGATCAACT
P5NEXTPT5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7_CTCTCTAC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8_CAGAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9_GCTACGCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10_CGAGGCTG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT	CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT

DNA & RNA extraction from fixed nuclei

1 Preheat a ThermoMixer to 55°C.

2 Pellet sorted nuclei in a 1.5 mL tube at 2500 x g, 4°C for 3min. 5m
Resuspend pellet in 1X ProtK digestion buffer (95 µL nuclease free water, 95 µL 2X digestion buffer, 10 µL Proteinase K)

3 Incubate at 55°C, 600 rpm for 1h. (Proteinase K digestion) 1h
In the meantime, preheat another ThermoMixer to 65°C.

4 Incubate at 65°C, 600 rpm for 15min. (Reverse Crosslinking) 15m

5 Centrifuge at maximum speed for 3min. (Get rid of cell debris) 5m
In the meantime, set the 2 ThermoMixers to 37°C and 26°C, respectively.

6 Transfer supernatant (190 µL) to a new tube, add 190 µL RNA/DNA lysis buffer (from Quick-DNA/RNA Microprep Plus Kit) and mix thoroughly. 5m

7 Continue with the Quick-DNA/RNA Microprep Plus Kit following the manufacturer's protocol. 30m
Perform in-column DNase digest for the RNA samples.

8 For **DNA** samples:
- Elute in 21 µL nuclease free water.
- Measure DNA concentration with the Qubit™ dsDNA HS Assay Kit, using 1 µL DNA.
- Freeze at -20°C for later use.

9 For **RNA** samples:
- Elute in 15 µL nuclease free water.
- Continue with TURBO DNase digestion and Reverse Transcription.

TURBO DNase digest and Reverse Transcription 1h 40m

10 Add to each RNA sample: 20m
- 1.5 µL TURBO DNase buffer (10X)
- 1 µL TURBO DNase

Incubate at 37°C, 600rpm for 20min

11 Add 2 µL inactivation reagent to each sample. 5m
Incubate at 26°C, 1600rpm for 5min.

12 ⚠

Transfer each sample to 0.5 mL tubes (critical, allows better aspiration of supernatant).
Centrifuge at 10'000 x g for 90s.

13 Aspirate the supernatant (15 µL). Do not take up any inactivation reagent!
Measure RNA concentration with the Qubit™ RNA HS Assay Kit, using 1 µL RNA.

14 ⊛

Optional: Determine the RIN value by analyzing the RNA samples with the RNA 6000 Pico Kit, following the manufacturer's protocol.

15 Use 12 µL RNA for Reverse Transcription. For that, set up the following reaction in 0.2 mL PCR tube:
- 12 µL RNA
- 1.5 µL dNTP mix
- 1.5 µL Oligo(dT)18 Primer

16 Incubate in a thermocycler with heated lid: 5m

- 5min at 65°C

17 Place reaction on ice for 1min.
Add 15 µL 2X RT mix to each sample (6 µL 5X RT buffer, 7.5 µL nuclease free water, 1 µL Maxima H- RT, 0.5 µL RNasin plus).

18 Incubate in a thermocycler with heated lid: 40m
- 30min at 50°C
- 5min at 85°C

19 30m

Transfer each sample to a DNA low binding 1.5 mL tube.
Clean up the cDNA with 1.5X vol. (= 45 µL) of AMPure XP magnetic beads following the manufacturer's protocol.
Elute the cleaned cDNA in 21 µL nuclease free water.
Freeze cDNA at -20°C for later use.

Attaching UMIs

20 In the two subsequent sections, DNA sequencing libraries are generated from both DNA and cDNA (RNA) samples. This is done in the exact same way for both sample types.

In this first PCR, UMIs are attached by 3 PCR cycles. Primer binding sites are in the C-terminal part of mScarlet-I (FWD_mScar_Tn7_10UMI_3) and immediately downstream of the 12bp barcode (RV_univ_MPRA). The fragment created in this PCR has a length of 196bp.

Set up the following PCR reaction:
- 17 µL DNA/cDNA from step **go to step #8** and step **go to step #19**, respectively
- 3 µL nuclease free water
- 2.5 µL primer FWD_mScar_Tn7_10UMI_3 (10µM stock)
- 2.5 µL primer RV_univ_MPRA (10µM stock)
- 25 µL NEBNext Ultra II Q5 Master Mix

> Don't use all 20 µL of cDNA: Leave 3 µL of cDNA for qPCR on marker genes.

21 Incubate in a PCR cycler with heated lid:
- 30s - 98°C
- 3 cycles (10s - 98°C; 30s - 65°C; 1min - 72°C)
- 5min - 72°C
- hold - 4°C

22 Transfer each sample to a DNA low binding 1.5 mL tube.
Clean up each sample with 1.2X vol. (= 60 µL) of AMPure XP magnetic beads following the manufacturer's protocol.
Elute in 20 µL nuclease free water.

Library amplification

23 The library amplification step is split up in 2 separate PCRs. In the first PCR, the P7 illumina adapter is added.

Then, in the remaining cycles, also the P5 illumina adapter is added. By splitting up the library amplification in two PCRs (with a total of 20-30 cycles, depending on input), the likelihood of primer dimer formation is drastically reduced in low-input samples.

Set up the following PCR reaction:

- 20 µL sample from step **go to step #22**
- 2.5 µL primer Ad2.X_index (2µM stock)
- 2.5 µL primer RV_univ_MPRA (2µM stock)
- 25 µL NEBNext Ultra II Q5 Master Mix

24 Incubate in a PCR cycler with heated lid:

- 30s - 98°C
- X cycles (10s - 98°C; 90s - 65°C)
- 5min - 65°C
- hold - 4°C

X cycles: 10 for DNA samples, 12 for cDNA samples

25 Transfer each sample to a DNA low binding 1.5 mL tube.
Clean up each sample with 1.2X vol. (= 60 µL) of AMPure XP magnetic beads following the manufacturer's protocol.
Elute in 10 µL nuclease free water.

26 Use 1.5 µL sample from step **go to step #25** for qPCR to estimate the remaining PCR cycles.

Set up the following qPCR reaction:

- 1.5 µL sample
- 0.5 µL primer Ad2.X_index (2µM stock)
- 0.5 µL primer P5NEXTPT5 (2µM stock)
- 2.5 µL nuclease free water
- 5 µL 2X Luna Universal qPCR Master Mix

27 Incubate in a Real-Time thermocycler:

- 5mins - 95°C
- 45 cycles (10s - 98°C; 10s - 60°C; 20s - 72°C)
- Cooling

28 ⚠

Determine the cycle number, where the amplification curve was at its half maximum.
Subtract 1 from this number. This is the required remaining cycle number.

29 Set up the following PCR reaction:

- 8 µL sample from step **go to step #25**
- 12 µL nuclease free water
- 2.5 µL primer Ad2.X_index (2µM stock)
- 2.5 µL primer P5NEXTPT5 (2µM stock)
- 25 µL NEBNext Ultra II Q5 Master Mix

30 Incubate in a PCR cycler with heated lid:

- 30s - 98°C
- X cycles (10s - 98°C; 90s - 65°C)

- 5min - 65°C
- hold - 4°C

X cycles: number determined in step go to step #28

31 Transfer each sample to a DNA low binding 1.5 mL tube.
Clean up each sample with 0.8X vol. (= 40 µL) of AMPure XP magnetic beads following the manufacturer's protocol.

The lower AMPure XP beads ratio here gets rid of potential primer dimers.

Elute in 10 µL nuclease free water. This is the final library.

32 Quantify by measuring 1 µL with the Qubit™ dsDNA HS Assay Kit.
Analyze the final library with the High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer.

The final library should have a size of 269bp.

CRE-barcode association

33 In this section, the DNA sequencing library for the CRE-barcode association is created. For that, the **MPRA pre-pool** (without min.Promoter_mScarlet-I) is used as input.

Set up the following PCR reaction:
- 5 ng MPRA pre-pool
- to 20 µL: nuclease free water
- 2.5 µL primer FWD_CRS_Tn7 (10µM stock)
- 2.5 µL primer RV_univ_MPRA (10µM stock)
- 25 µL NEBNext Ultra II Q5 Master Mix

34 Incubate in a PCR cycler with heated lid:
- 30s - 98°C
- 3 cycles (10s - 98°C; 30s - 65°C; 3min - 72°C)
- 5min - 72°C
- hold - 4°C

The longer extension time prevents chimeric DNA annealing caused by incomplete elongation.

35 Transfer the sample to a DNA low binding 1.5 mL tube.
Clean up the sample with 1.2X vol. (= 60 µL) of AMPure XP magnetic beads following the manufacturer's protocol.
Elute in 20 µL nuclease free water.

36 Set up the following PCR reaction:
- 20 µL PCR product
- 2.5 µL primer Ad2.X_index (2µM stock)

- 2.5 µL primer P5NEXTPT5 (2µM stock)
- 25 µL NEBNext Ultra II Q5 Master Mix

37 Transfer the sample to a DNA low binding 1.5 mL tube.
Clean up the sample by double size selection with 0.5X vol. and 0.8X vol. of AMPure XP magnetic beads following the manufacturer's protocol.
Elute in 10 µL nuclease free water.

38 Quantify by measuring 1 µL with the Qubit™ dsDNA HS Assay Kit.
Analyze the final library with the High Sensitivity DNA Kit on the Agilent 2100 Bioanalyzer.

The final library should have a size of 455bp.