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MAPseq (Multiplexed Analysis of Projections by Sequencing) sample processing protocol

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1 Works for me dx.doi.org/10.17504/protocols.io.bsm9nc96

Cold Spring Harbor Laboratory | The MAPseq Core_CSHL

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ABSTRACT

This protocol describes sample processing steps of MAPseq, a high-throughput mapping of single-neuron projections by sequencing of barcoded RNA, as described in details by Kebschull et al.., 2016 . In MAPseq, a brain area of interest is infected with a Sindbis barcoded library. After 40-44 hrs, the injection and the projection sites of interest are dissected, processed and sequenced using this protocol.

EXTERNAL LINK

https://pubmed.ncbi.nlm.nih.gov/27545715/

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Kebschull JM, Garcia da Silva P, Reid AP, Peikon ID, Albeanu DF, Zador AM. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. Neuron. 2016 Sep 7;91(5):975-987. doi: 10.1016/j.neuron.2016.07.036. Epub 2016 Aug 18. PMID: 27545715; PMCID: PMC6640135.

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PROTOCOL CITATION

Huiqing Zhan, Justus Kebschull, Anthony M. Zador 2021. MAPseq (Multiplexed Analysis of Projections by Sequencing) sample processing protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bsm9nc96

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Kebschull JM, Garcia da Silva P, Reid AP, Peikon ID, Albeanu DF, Zador AM. High-Throughput Mapping of Single-Neuron Projections by Sequencing of Barcoded RNA. Neuron. 2016 Sep 7;91(5):975-987. doi: 10.1016/j.neuron.2016.07.036. Epub 2016 Aug 18. PMID: 27545715; PMCID: PMC6640135.

KEYWORDS

MAPseq, Sequencing of Barcoded RNA, Mapping single neurons

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GUIDELINES

Extra caution should be taken to avoid cross sample contamination during RNA extraction and reverse transcription.
Use PCR II test to decide the optimal PCR II cycles.
Try to use the least PCR II cycle to minimize the template switching rate.

MATERIALS TEXT

Trizol reagent Thermo Fisher 15596018
Superscript IV Thermo Fisher 18090010
Second Strand cDNA Synthesis Kit, Thermo Fisher A48571
AMPure XP Beckman Coulter A63881
Exonuclease I (E. coli) NEB M0293S
RNasin Ribonuclease Inhibitor Promega N2611
AccuPrime™ Taq DNA Polymerase, high fidelity. Thermo Fisher 12346086
Wizard® SV Gel and PCR Clean-Up System Promega A9282
Agilent High Sensitivity DNA Kit 5067-4626
Qiagen MinElute Gel Extraction Kit 28606
RT primer sequence: CTT GGC ACC CGA GAA TTC CAN NNN NNN NNN NNX XXX XXX XTG TAC AGC TAG CGG TGG TCG, where N-12 is the UMI and X-8 is the SSI.
Spike-in RNA sequence: GTC ATG ATC ATA ATA CGA CTC ACT ATA GGG GAC GAG CTG TAC AAG TAA ACG CGT AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC TNN NNN NNN NNN NNN NNN NNN NAT CAG TCA TCG GAG CGG CCG CTA CCT AAT TGC CGT CGT GAG GTA CGA CCA CCG CTA GCT GTA CA, where ATCAGTCA is the barcode tag of the spike-in. Spike-in RNA was transcribed in vitro by T7 RNA polymerase and diluted into 10^3 molecules/ul for target sites and 10^5 molecules/ul for injection sites.
Nested1st gfpF primer sequence: CTG TAC AAG TAA ACG CGT AAT G
Nested2nd R primer sequence: CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CCT TGG CAC CCG AGA ATT CCA
Sol I primer sequence: AAT GAT ACG GCG ACC ACC GA
Sol II primer sequence: CAA GCA GAA GAC GGC ATA CGA

DISCLAIMER:

RNA extraction

1 Each fresh frozen brain area of interest is dissected and kept in RNAse-free tubes at -80°C before RNA extraction.

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2 Tissues are homogenized in 400ul of Trizol either with a Pellet Pestle Motor or with a tissue homogenizer, and then add 600ul of Trizol to make 1 ml of Trizol/sample
Extract RNA according to manufacturer's protocol of Trizol Reagent
Dissolve RNA of each tissue sample into 13 ul of H2O

Reverse transcription with Superscript IV

3 One reaction per sample.
For each reaction, add the following reagents(total of 13ul) into each well:
4 ul of RNA from each sample
6 ul of H2O
1 ul of spike-in RNA (Spike-in RNA has its unique barcode and is used to normalize for varying reverse transcription, PCR or library making efficiencies)
1ul of 10uM RT primer (each RT primer contains a random 12-nt unique molecular identifier (UMI) to tag each individual barcode mRNA molecule and a 8-nt slice specific identifier (SSI) to tag each sample)
1 ul of 10mM dNTP
Mix well.

4 Incubate at 70 °C for 10 minutes, then immediately transfer samples to ice.
Incubate on ice for 5 minutes.

5 Add per well (total of 7ul)
4ul SSIV buffer
1ul 0.1M DTT
1ul RNasin
1ul SSIV
Mix well.

6 Incubate the mixture in a thermocycler
10min at 55 °C
10min at 80 °C
Store samples at 4 °C or -20ºC till next step.

AMPure XP beads clean up

7 Pool all of the RT product from targets or injection sites.
Do not mix target site with injection site.
Mix well.

8 For pooled samples from target sites
Add 1.8X AMPure XP beads
Mix by pipetting 10 times
Incubate for 10 minutes at room temperature
Aliquot into several tubes for easier elution
Put on magnetic rack, wait for 2-3 minutes till solution is clear
On magnetic rack, discard supernatant
Wash twice with fresh 80% EtOH to cover the pellet for 30 seconds
Air dry pellet till
Resuspend beads in (17 * (total number of target site/10) ul) of H20
Incubate at room temperature for 5 minutes
On magnetic rack, collect all of supernatant in an Eppendorf tube.

9 For pooled samples from injection sites
Add 1.8X AMPure XP beads
Mix by pipetting 10 times

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Incubate for 10 minutes at room temperature
Aliquot into several tubes for easier elution
Put on magnetic rack, wait for 2-3 minutes till solution is clear
On magnetic rack, discard supernatant
Wash twice with fresh 80% EtOH to cover the pellet for 30 seconds
Air dry pellet
Resuspend beads in (17 * (total number of target site/5) ul) of H20
Incubate at room temperature for 5 minutes
On magnetic rack, collect all of supernatant in an Eppendorf tube.

2nd strand cDNA synthesis

10 Per 17ul of bead purified product, add
2.4ul SSIV buffer
0.6ul 0.1M DTT
5.6ul Second Strand buffer
0.75ul 10mM dNTPs
0.25ul E coli DNA ligase
1ul DNA polymerase I
0.25ul RNaseH
Mix well.

11 Incubate the mixture in a thermocycler
2 hours at 16ºC
Add 1ul of T4 DNA polymerase
Incubate for 10 minutes at 16ºC.
Store sample at 4ºC or -20ºC till next step.

Beads clean up

12 Pool all 2nd stranding reactions, measure the total volume
Add 1.8x AMPure XP beads
Mix by pipetting 10 times
Incubate for 10 minutes at room temperature
Put on magnetic rack, wait for 2-3 minutes till solution is clear
On magnetic rack, discard supernatant
Wash twice with fresh 80% EtOH to cover the pellet for 30 seconds
Air dry pellet
Resuspend beads in (16 * (total number of 2nd strand reaction) ul) of H20
Incubate at room temperature for 5 minutes
On magnetic rack, collect all of supernatant in a PCR tube.

Exonuclease treatment

13 Per 16ul of beads purified product, add
2ul Exonuclease buffer
2ul Exo I
Mix well.

14 Incubate the mixture in a thermocycler
1 hr at 37 ºC
Heat inactivate at 80 ºC for 20 minutes
Store sample at 4ºC or -20ºC till next step.

PCR I

15 Per 20ul Exonuclease reaction, add
25ul Accuprime buffer
25ul 10uM nested1st gfpF primer

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25ul 10uM nested2nd primer
2.5 ul Accuprime Pfx HF enzyme
152.5ul H20
Mix well and aliquot the mixture into PCR tubes

16 Perform thermal cycling as follows:
Initial denaturation at
95ºC 2 minutes
Run 15 cycles of
95ºC 15 seconds
68ºC 2.5 minutes
Final extension at
68ºC 5 minutes

Exonuclease treatment

17 Add 5 ul of Exo I to each 50 ul PCR reaction
Incubation at 37ºC for 30 minutes
Heat inactivation at 80ºC for 20 minutes

Test PCR II

18 Test optimal PCR II cycle number in a 25ul reaction/test by adding
2.5ul of 1/10 diluted PCR I product
2.5ul Accuprime buffer
2.5ul 10uM Sol I primer
2.5ul 10uM Sol II primer
0.25ul Accuprime Pfx HF enzyme
14.75ul H20

19 For pooled target sites, run 14, 17, 20, 23, 26 and 29 cycles
For pooled injection sites, run 11, 14, 17, 20, and 23 cycles
Perform thermal cycling as follows:
Initial denaturation at
95ºC 2 minutes
Run different cycles of
95ºC 15 seconds
68ºC 1 minutes
Final extension at
68ºC 5 minutes

20 Load test PCR II products on 2% agarose gel to find out the lowest cycle number which could generate a single and clean 230bp band.

PCR II

21 For pooled injection sites, use 12 ml of PCR II reaction
For pooled target sites, use 3 ml of PCR II reaction for <12 samples, 6 ml for <40 samples, and 12 ml for >40 samples.

22 In each PCR II reaction,
Add the same concentration of PCR I product as what is used in the test PCR II
1x Accuprime buffer
1uM Sol I primer
1uM Sol II primer
1U of Accuprime Pfx HF enzyme/50ul of reaction

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23 Perform thermal cycling as follows:
Initial denaturation at
95ºC 2 minutes
Run optimal cycles of
95ºC 15 seconds
68ºC 2.5 minutes
Final extension at
68ºC 5 minutes

Gel purification

24 Purify PCR product using Wizard® SV Gel and PCR Clean-Up System from Promega and elute the product into 40ul of H2O per 1ml of PCR product

25 Load purified PCR product into 2% agarose gel

26 Cut ~230bp band from the gel and purify the product with MinElute Gel Extraction Kit from Qiagen

Bioanalyzer test with Agilent High Sensitivity DNA Kit

27 Load purified 230bp PCR product on a DNA bioanalyzer chip using Agilent High Sensitivity DNA Kit to confirm its size and quantity before submitting for sequencing

Next Generation Sequencing (NGS)

28 Submit purified 230bp product for an Illumina NextSeq500 high output run at paired end 36 using the SBS3T sequencing primer for paired end 1 and the Illumina small RNA sequencing primer 2 for paired end 2.

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