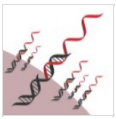

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Multi-Seq: my notes from the lab

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

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ABSTRACT

Please note these are just my notes and by no means are ground truth of how Multi-seq needs to be performed. I WELCOME to comments, suggestions and amendments.

GUIDELINES

Notes are for nuclei/CMO; however, for cells/LMO the protocol is the basically the same but using 0.04% BSA because BSA sequesters LMOs (See Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019).

You may scale up or down LMO/CMO concentrations while maintaining the same ratio LMO/CMO per cell/nuclei used in paper.

-For LMOs/CMOs on live cells that's 8 pmol/100K or 40 pmol/500K, in a reaction volume of 200 uL, which gives you 40 nM (100K cells) and 200 nM (500K cell), respectively.

-For LMOs/CMOs on nuclei they seem to have used 500 uM (500K nuclei), that is 100 pmol/500K, in (likely) a reaction volume of 200 uL, so it'll be 20 pmol/100K nuclei (this is 2.5 times higher than for lives cells). We have used CMOs at 200 nM in a 200 uL reaction and works fine. Both version will be given in the step-by-step protocol, but the one inferred from paper (using 500 nM) will be *[in between brakets and italized].*

-I would recommend to determine what's the optimal amount of CMO or LMO/number of cells or nuclei in your hands. For GM12878 cell line I used 200 nM for nuclei and managed to get very good libraries. You may also want to test 200 nM versus 500 nM and see which is best for your sample. From Supplementary Figure 1, panels A and C, 200 nM seems to be enough.

-We have also replaced polyA for Capture sequence 2 (Feature), libraries look very good as well. This was done to avoid competition between CMO/LMOs and endogenous RNAs for polyA oligos.

Sequence below:

5'-CCTTGGCACCCGAGAATTCCA**XXXXXXXX**GCTCACCTATTAGCGGCTAAGG-3'

MATERIALS TEXT

Citation: Luciano Martelotto (04/22/2020). Multi-Seq: my notes from the lab. https://dx.doi.org/10.17504/protocols.io.bejyjcpw

Materials are those in Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019. You will also need either DNA binding dies like DAPI, 7-AAD or DRAQ-7 if you are using FANS. For sucrose gradient use this NUC201-1KT (Sigma).

SAFETY WARNINGS

Every below step was done 4°C.

BEFORE STARTING

-I strongly recommend to read Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019.

-Multi-seq is a great tech and not difficult, and yet it needs a bit optimisation to get it to work consistently. Chris and David are amazingly helpful blokes and always keen to help if you have any questions. Don't hesitate in getting in touch...I did, a lot!

-Be mindful these are just my notes and by no means are ground truth of how Multi-seq needs to be performed. I WELCOME to comments, suggestions and amendments.

1 First thing to do is to get **very clean nuclei preps**. You may start with less clean nuclei if you like but it is likely that CMO will bind to the debris, and that is not good (just a thought). So, if you work with cell lines, iPSCs, PBMCs or some of the 'liquid' cancers then nuclei will be pretty clean, but depending on the solid tissues, as you probably have experienced already, you may end up getting dirty nuclei preps.

2 To get clean nuclei you can use either a sucrose gradient or FANS based on DNA content staining DAPI/7-AAD/DRAQ-7(*). I prefer FANS (but this is just me and because I have the sorter in the lab), and for most snRNA-Seq experiments I use DAPI or 7-AAD or DRAQ-7 (I used DAPI more often). After FANS nuclei usually looks super clean and start from there. Sucrose gradient works also quite well, specially to remove myelin from brain tissue, but haven't used it regularly enough. In any case, starting with clean nuclei (when possible) is good practice to try to have the best and reproducible labelling as possible.
(*) These are also dyes are known to not distort chromatin (or very little, as opposed to PI which it does) so we keep this door open for when we do snRNA and snATAC from same sample.
Note: FANS based on DNA content is not just an expensive way to clean up nuclei, it can help you identify potential sub-polulation with different ploidy, in particular for cancer samples.

3 It is important that you visually inspect the nuclei preps under the optical microscope (brightfield) before and after sorting. Check for signs of nuclei damage or partially lysed cells. Nuclei sizes and shape vary a lot, so I don't take this too much into account. I do look at the edges of the nuclei - usually intact nuclei have clear edges. Damaged nuclei are easy to identify with 40x-60x magnification. Sometimes disorganised chromatin popping our out of the nucleus is visible, which is bad sign. Nuclei blebbing is also bad sign.

4 Mix Anchor and Barcoded oligos in 1:1 molar ratio in PBS (no Ca^{2+}/Mg^{2+}) at **0.8 Micromolar (µM)** (*), that is 10 times solution (of the duplex) and incubate at **Room temperature** for **00:05:00** . We have been preparing this mix fresh. (*) The working concentration is optional.

[Mix Anchor and Barcoded oligos in 1:1 molar ratio in PBS (no Ca^{2+}/Mg^{2+}) at **2 Micromolar (µM)** *(*), that is 10 times solution (of the duplex) and incubate at* **Room temperature** *for* **00:05:00** *. We have been preparing this mix fresh. (*) The working concentration is optional.]*

What (I think) it's key is to maintain the ratio **40 pmol** *[or* **100 pmol** *]* of CMOs (Anchor-Barcoded Oligo and Co-Anchor) per 500,000 cells (**200 Nanomolar (nM)** *[or* **500 Nanomolar (nM)** *]*) final in **200 µl**). So for 5x less nuclei, namely 100,000 nuclei, we use **8 pmol** *[or* **20 pmol** *]* CMO, which is 5x less.

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5 The number of nuclei you use of of your choice, for example ~100,000 nuclei(*) in Washing Buffer [PBS (no Ca2+/Mg2+) + **1 %** BSA (MACS BSA Stock Solution from Miltenyi, Cat. 130-091-376)]. Note: remember that for cells you use **0.04 %** BSA or no BSA in your Washing Buffer.
If FANS is not an option, then directly resuspend nuclei in 180 uL Washing Buffer and continue with Step 7 below.
We usually chose FANS for our nuclei preps clean up step prior to snRNA-Seq (see *Frankenstein* protocol for snRNAseq using fresh and frozen samples on 10x Platform). (*)Our sorter offset is ~40%, so we sort ~140,000 events to get ~100,000 nuclei.
The sorting is done in **50 µl** of Washing Buffer in 96-well plate (culture plate, round bottom, but LoBind **1.5 ml** eppis work too). I like culture plates because I always inspect nuclei under the microscope after sorting, so these are ideal.

6 After sorting, transfer the nuclei to a **1.5 ml** LoBind eppis tube and complete with chilled Wash Buffer to ~ **180 µl** **1.5 ml** (Note: when limited nuclei number, I usually use the additional volume to 'wash' the well where the nuclei come from and then transfer this wash the respective nuclei in the eppi).

7 Add **8 pmol** Anchor-Barcode oligo (**8 pmol** = **10 µl** of **0.8 Micromolar (µM)**) and gently but thoroughly mix by pipetting using wide-bore tip. So far we've maintained the **40 pmol** CMO/500,000 nuclei (**8 pmol** CMO/100,000, that's 5x less). For 500,000 nuclei add **20 µl** of **2 Micromolar (µM)** CMO for a final concentration of **200 Nanomolar (nM)** in **200 µl** final volume).
Here I am accounting for 5x less cells, so I add **10 µl** of **0.8 Micromolar (µM)** CMO in a final volume of ~ **190 µl** (Note: at this stage the Anchor-Barcoded oligo concentration is **42.105 Nanomolar (nM)** , but it'll become **40 Nanomolar (nM)** when I add the Co-Anchor below (bringing the final volume from **190 µl** to **200 µl**). In any case, for 100,000 nuclei Anchor-Barcoded Oligo and Co-Anchor will at the same molarity in the end, that is **40 Nanomolar (nM)** or **8 pmol** each in **200 µl** final volume, which is 5x less of what's used for 500,000 nuclei, namely **200 Nanomolar (nM)**).

[Add **20 pmol** *Anchor-Barcode oligo (* **20 pmol** *=* **10 µl** *of* **2 Micromolar (µM)** *) and gently but thoroughly mix by pipetting using wide-bore tip. So far we've maintained the* **100 pmol** *CMO/500,000 nuclei (* **20 pmol** *CMO/100,000, that's 5x less). For 500,000 nuclei add* **20 µl** *of* **2 Micromolar (µM)** *CMO for a final concentration of* **500 Nanomolar (nM)** *in* **200 µl** *final volume).*
Here I am accounting for 5x less cells, so I add **10 µl** *of* **2 Micromolar (µM)** *CMO in a final volume of ~* **190 µl** *(Note: at this stage the Anchor-Barcoded oligo concentration is* **105.26 Nanomolar (nM)** *, but it'll become* **100 Nanomolar (nM)** *when I add the Co-Anchor below (bringing the final volume from* **190 µl** *to* **200 µl** *). In any case, for 100,000 nuclei Anchor-Barcoded Oligo and Co-Anchor will at the same molarity in the end, that is* **100 Nanomolar (nM)** *or* **20 pmol** *each in* **200 µl** *final volume, which is 5x less of what's used for 500,000 nuclei, namely* **500 Nanomolar (nM)** *).]*

8 Incubate for **00:05:00** at **4 °C** .

9 Add **8 pmol** *[or* **20 pmol** *]* Co-Anchor and gently but thoroughly mix.

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10 Incubate for **00:05:00** at **4 °C** .

11 Add **1 ml** of chilled Wash Buffer and mix by inverting 3x.

12 **500 x g, 4°C 00:05:00**

13 Remove supernatant without disturbing the pellet. For this, I always leave behind ~ **20 µl** - **30 µl** of supernatant. This is key to avoid losing the nuclei.

14 Repeat wash for 2 more times using **1.2 ml** of chilled Wash Buffer. **Do not resuspend** nuclei after every wash – this help reducing nuclei loss.

15 In the final wash resuspend in **80 µl** of chilled Wash Buffer supplemented with **0.2 U/µl** - **0.5 U/µl** of RNAse Inhibitor (Protector RNAse Inhibitor, Cat. RNAINH-RO, Merck/Roche). One may use RNAse inhibitor all the way, but it becomes expensive. I use RNAse Inhibitor during cell lysis, and in this final resuspension and RNA/cDNA quality looks good.

16 If possible, count using Countess II Automated Cell Counter using Trypan Blue (tend to precipitate so it's a pain. I spin top speed every time I use) or Ethidium Homodimer. Alternatively, proceed with pooling.

17 Combine nuclei from multiple samples to multiplex and filter with a 40-um Flowmi® Cell Strainer.

18 Count using Countess II Automated Cell Counter.

19 Adjust nuclei concentration if needed.

20 Proceed with 10x 3' v3 protocol.

21 After GEM formation and RT proceed as per 3' v3.x workflow until cDNA amplification. Elute cDNA in **35 µl** .

22 Transfer **34 µl** of cDNA onto cDNA Amplification mix supplemented with **1 µl** of **2.5 Micromolar (µM)** of Multi-Seq Additive primer.

23 Perform cDNA amplification and 0.6X SPRI clean-up according to 3' v3.x workflow.

24 Endogenous transcript cDNA is bound to the SPRI beads. Continue protocol for endogenous transcripts without change.

25 Add 1.4X SPRI to supernatant to obtain a final SPRI volume of 2X SPRI. For example, the cDNA PCR reaction volume is **100 µl** , therefore add another **140 µl** of SPRI to the supernatant which already contains 60 µl of SPRI. The final volume will be **300 µl** containing 2X SPRI. Transfer entire volume into a low-bind 1.5 mL tube and Incubate for **00:05:00** at room temperature.

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26 Place tube on magnet and wait ~ **00:02:00** until solution is clear and then carefully remove and discard the supernatant.

27 Add 400 µl 80% Ethanol to the tube without disturbing the pellet and stand for **00:00:30** (only one 80% Ethanol wash). Carefully remove and discard the ethanol wash.

28 Centrifuge tube briefly and return it to magnet. Remove and discard any remaining ethanol.

29 Resuspend in beads in **50 µl** water.

30 Perform another round of 2X SPRI purification by adding **100 µl** SPRI reagent directly onto resuspended beads. Mix by pipetting and incubate **00:10:00** at room temperature.

31 **00:02:00** Place tube on magnet and wait ~ **00:02:00** until solution is clear. Carefully remove and discard the supernatant.

32 Add **200 µl** 80% Ethanol to the tube without disturbing the pellet and stand for **00:00:30** (1st Ethanol wash). Carefully remove and discard the ethanol wash.

33 Add **200 µl** 80% Ethanol to the tube without disturbing the pellet and stand for **00:00:30** (2nd Ethanol wash). Carefully remove and discard the ethanol wash.

34 Centrifuge tube briefly and return it to magnet. Remove and discard any remaining ethanol and allow the beads to air dry for **00:02:00** . **Do not over dry beads.**

35 Resuspend beads in **40 µl** water or EB. Pipette mix vigorously and incubate at room temperature for **00:02:00** .

36 Place tube on magnet and transfer clear supernatant into one PCR tube.

Quantification of barcode DNA concentration using Qubit (typical range is 0.5 - 5 ng/µL) is recommended but it's optional.

-If quantifying the use **3.5 ng** of material for indexing PCR.

-Alternatiovely use **5 µl** of material for indexing.

-The use of the whole material is also possible but some primer optimization may be needed as you PCR bubbles due to over-cycling is likely (i.e. primers get exhausted). When this happen, run a P5/P7 reaction for one-2 cycles.

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37 Set up the PCR reaction as follows:

50 µl Kapa HiFi HotStart ReadyMix (2X)

5 µl 10 µM SI Primer (e.g. 3' v3.1, PN 2000095)

5 µl 10 µM RPIx primer (choose unique RPI for each sample from 10X lane)

3.5 ng barcode DNA or **5 µl** or entire volume (~ **40 µl**)

Nuclease-free water to **50 µl** final volume.

Cycling conditions:

1. **95 °C** x **00:05:00**
2. **98 °C** x **00:00:20**
3. **60 °C** x **00:00:30**
4. **72 °C** x **00:00:30**

Go to 2 for ~6-10 cycles

5. **72 °C** x **00:05:00**
6. Hold at **4 °C** .

38 Add **160 µl** (1.6X) SPRI to each PCR product, pipette mix thoroughly. Incubate at room temperature for **00:05:00** .

39 Place tube on magnet (High), wait for solution to clear. Remove and discard supernatant.

40 Wash beads twice on magnet (High) with **200 µl** of 80% ethanol and allow to stand for **00:00:30** between washes.

41 After second wash, briefly centrifuge beads and place on magnetic rack (Low). Remove remaining ethanol with P20 micropipette. Air-dry beads on magnet for **00:02:00** . **Do not over dry beads.**

42 Remove from magnet, resuspend beads in **25 µl** buffer EB and pipette mix thoroughly to resuspend. Incubate at room temperature for **00:02:00** .

43 Return to magnet (Low), wait for solution to clear, and transfer supernatant to PCR strip tube.

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44 Quantify barcode library concentration (1:5 - 1:10) using Bioanalyzer High Sensitivity DNA analysis. See representative electropherogram below.

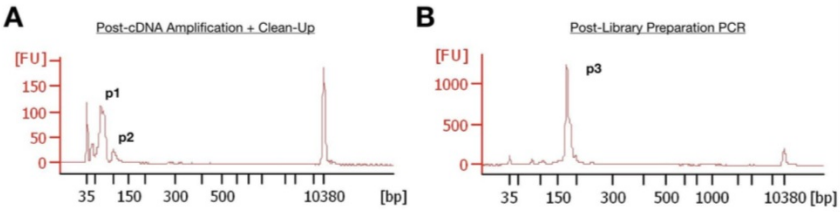

Figure 1. This is part of Supplementary Figure 9 of paper:
(A) Bioanalyzer traces following cDNA amplification and MULTI-seq barcode enrichment using 3.2X SPRI with 1.8X 100% isopropanol exhibits two distinct peaks. Bioanalyzer traces are representative of all datasets presented in this study (n = 4). The first peak (p1) is an average of 65-70bp in length and likely corresponds to barcodes amplified via the MULTI-seq additive primer. The second peak (p2) is an average of 100bp in length and likely corresponds to barcodes that successfully underwent MMLV-RTase template switching and were subsequently amplified by the standard 10X Genomics Single Cell V2 primer.
(B) Bioanalyzer analysis following library preparation PCR exhibits one distinct peak (p3) with an average length of 173bp, matching expectations. Bioanalyzer traces are representative of all datasets presented in this study (n = 4).

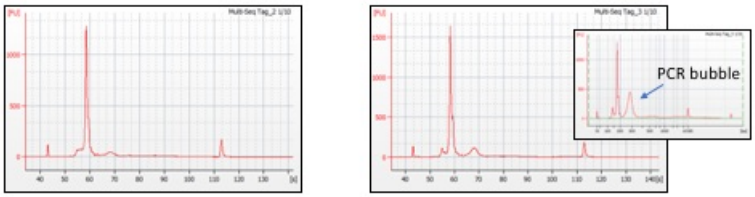

Figure 2. Bioanalyzer traces. These libraries were prepared using the entire volume from step 36. Left panel inlet shows PCR bubble.

45 Barcodes can be sequenced independently or as fraction of endogenous cDNA library. Aim for ~3000-5000 read pairs per cell.

46 For analysis go to https://github.com/chris-mcginnis-ucsf/MULTI-seq.

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