**Gene-expression profiling by Smart-3SEQ**

**U01 MCL PCA Supplemental Project**

**Version 1.4 – 11/23/2018**

*This SOP is adapted from the Joseph W. Foley / Robert West* [*Protocol*](https://www.biorxiv.org/content/biorxiv/suppl/2018/07/24/207340.DC3/207340-2.pdf) *- Department of Pathology Stanford University School of Medicine, which should be used for reference. This version of the protocol is optimized for formalin-fixed, paraffin-embedded tissue sections, laser capture micro-dissected on an Arcturus HS LCM Cap (*[*Thermo-Fisher LCM0214*](https://www.thermofisher.com/order/catalog/product/LCM0214)*) and recommends pooled clean-up of multiple libraries. FFPE tissues require longer digestions time and result in highly degraded RNA. Please read entire protocol before starting.*

1. Pre-warm an incubator at 60 °C with the metal HS CapSure incubation block (Thermo-Fisher [LCM0213](https://www.thermofisher.com/order/catalog/product/LCM0213) or [LCM0505](https://www.thermofisher.com/order/catalog/product/LCM0505)) inside. Note: It may help to place an open container of water in the incubator for humidity.

2. Aliquot 5 μL FFPE LCM Lysis Mix directly onto the in the center of the LCM HS Cap.

 **FFPE LCM Lysis Mix**

|  |  |  |  |
| --- | --- | --- | --- |
| **REAGENT**  | **CATALOG NUM.** | **Qty** | **MULTI (x )** |
| Betadine solution (5M) | [Sigma-Aldrich B0300](https://www.sigmaaldrich.com/catalog/product/sigma/b0300?lang=en&region=US) | 2.0 µl  |   |
| dNTP mix (10 mM each)  | [Thermo-Fisher R0192](https://www.thermofisher.com/order/catalog/product/R0192) | 1.0 µl  |   |
| First strand (1S) primer (20 uM) | IDT: Note 2 | 0.5 µl  |   |
| Triton-X-100 (0.5% v/v)  |   | 0.5 µl  |   |
| Proteinase K\* (0.125 ug/uL) | [NEB P8107S](https://www.neb.com/products/p8107-proteinase-k-molecular-biology-grade#Product%20Information) | 1 µl  |   |
|  | **FINAL Volume** | 5.0 µl  |   |

 \**Concentration of undiluted Proteinase K (800 units/ml) is 20 μg/μl. Dilute it 20 fold (to 1 ug/uL), then 8 fold (to 0.125 ug/uL) in Molecular Biology Water.*

3. Cut the cap of a 0.5 mL low-retention tube ([Thermo-Fisher N8010611](https://www.thermofisher.com/order/catalog/product/N8010611)) and place the tube directly onto the HS LCM cap. Avoid letting the droplet touch the tube, If it does, collect the entire droplet in the tube by brief centrifugation and try to put it back on the center of the cap. Seal tube with parafilm.

4. Place the upside-down cap and tube in the pre-warmed incubation block. Be careful to maintain the cap horizontal with the drop in the center.

5. Incubate 60 min at 60 °C.

6. When the incubation is complete, briefly centrifuge the tube (with cap) to collect the lysate, then remove the cap. You can inspect the cap under a microscope to verify complete lysis. Transfer supernatant to a new 0.2 µl low-retention tube or plate ([Eppendorf 0030603303](https://online-shop.eppendorf.us/US-en/Laboratory-Consumables-44512/Plates-44516/DNA-LoBind-Plates-PF-16858.html)).

7. Prepare FFPE LCM TS-RT Mix (on cold block/ice)

**LCM TS-RT Mix**

|  |  |  |  |
| --- | --- | --- | --- |
| **REAGENT** | **CATALOG NUM.** | **Qty** | **MULTI (x )** |
| SMARTScribe buffer, 5X | [Takarabio 639538](https://www.takarabio.com/products/cdna-synthesis/reverse-transcriptases/smartscribe) | 2.0 μL |   |
| Dithiothreitol (DTT, 20 mM) | [Takarabio 639538](https://www.takarabio.com/products/cdna-synthesis/reverse-transcriptases/smartscribe) | 1.0 µl  |   |
| RNase inhibitor (20 x)  | [Thermo-Fisher AM2694](https://www.thermofisher.com/order/catalog/product/AM2694) | 0.5 µl  |   |
| Second strand primer (2S, 50 uM) | IDT: Note 2 | 0.2 µl  |   |
| MgCl2 (200 mM)  | [Millipore-sigma M1028](https://www.sigmaaldrich.com/catalog/product/sigma/m1028?lang=en&region=US) | 0.2 µl  |   |
| Proteinase K inhibitor (5 mM in DMSO)  | [Millipore-Sigma 539470](http://www.emdmillipore.com/US/en/product/Proteinase-K-Inhibitor-Calbiochem%2CEMD_BIO-539470) | 0.1 µl  |   |
| SMARTScribe reverse transc. (100 U/ul) | [Takarabio 639538](https://www.takarabio.com/products/cdna-synthesis/reverse-transcriptases/smartscribe) | 1.0 µl  |   |
|   | **FINAL Volume** | 5.0 µl  |   |

8. Prepare(preheat) thermal-cycler with (Program 1: TS-RT Only)

|  |  |  |  |
| --- | --- | --- | --- |
| **Program 1: TS-RT** | **Temperature** | **Time (min)** | **Cycles** |
| 42 °C | hold | 1 |
| 42 °C | 30  | 1 |
| 70 °C | 10 | 1 |
| 4 °C | hold | 1 |

9. Place the tube (from step 6) in the thermal cycler, add 5 µl LCM TS-RT mix (step 7) and end the 42 °C hold.

10. After completion of Program 1. Transfer samples to cold block/ice

11. Prepare (preheat) thermal-cycler with (Program 2: PCR)

|  |  |  |  |
| --- | --- | --- | --- |
| **Program 2: PCR** | **Temperature** | **Time**  | **Cycles** |
| 98 °C |   | Hold | 1 |
| 98 °C |   | 45 sec | 1 |
|     | 98 °C | 10 sec | 12\* (**see note 4**) |
| 60 °C | 30 sec |
| 72 °C | 10 sec |
| 72 °C |   | 60 sec | 1 |
| 4 °C |   | hold | 1 |

12. Add 2.5 μL PCR primer mix with this library's unique combination of indexes (**see Note 3**), 2 μM each.

13. Add 12.5 μL HiFi HotStart ReadyMix (2X) ([Kapa KK2601](https://www.kapabiosystems.com/product-applications/products/pcr-2/kapa-hifi-pcr-kits/#accordion-order)), and mix by pipetting. Final volume should be 25 µl.

14. Place tube in thermal cycler (step 11) and start program 2.

15. When Program 2 reaches the 4 °C hold and the samples have cooled, briefly centrifuge the tubes (or plate).

16. Combine the samples into a single 1.5 mL (N ≤ 35 samples) or 2.0 mL (N ≤ 47) low-retention tube.

17. Add 17.5 μL of SPRI bead mix per sample (e.g. 35 samples = 612.5 L). Mix very well by pipetting.

18. Incubate the tube for 5 min at room temperature.

19. Place tubes on the magnet and wait for the beads to separate completely.

20. Without disturbing the pellet, remove and discard all the supernatant.

21. Remove the tube from the magnet and resuspend the pellet in 102 μL Re-SPRI Mix.

**RE-SPRI mix**

|  |  |  |  |
| --- | --- | --- | --- |
| **REAGENT** | **Cat** | **Qty** | **MULTI (X )**  |
| Molecular-biology grade water |  | 60 μL |  |
| AmpPure XP beads  | [Beckman Coulter A63880](https://www.beckman.com/reagents/genomic/cleanup-and-size-selection/pcr/A63880) | 42 µl  |  |
|  | **FINAL Volume** | 102 µl  |  |

22. Incubate the tube 5 min at room temperature.

23. Place tube on the magnet and wait for the beads to separate completely.

24. Without disturbing the pellet, remove and discard all the supernatant.

25. Still on the magnet, overlay the pellet with 1 ml freshly prepared 80% ethanol and wait 30 seconds.

26. Remove and discard the supernatant, then repeat the wash (step 24).

27. Remove and discard all the remaining supernatant. Use a smaller pipet to collect residual droplets.

28. Leave the tube open to air-dry for exactly 2 min. Do not over-dry the pellet or it will be difficult to resuspend.

29. Remove the tube from the magnet, thoroughly resuspend the pellet in 10 μL DNA Storage Buffer (briefly centrifuge the tube if necessary to collect the pellet) and wait 30 seconds.

**DNA storage buffer**

|  |  |  |
| --- | --- | --- |
| **REAGENT** | **Cat** | **Qty** |
| Molecular-biology grade water |   | 48.564 mL |
| Tris base (1 M) |   | 500 µl |
| disodium EDTA, 0.1 M |   | 500 µl |
| Tween 20, 10% (v/v) |   | 250 µl  |
| HCl, (1 M) |   | 186 µl  |
|   | **FINAL Volume** | 50 ml |

25. Return the tube to the magnet and allow the beads to separate completely.

26. Transfer the supernatant to a new tube.

27. Quantify by Fluorometer (Qbit) and/or Bioanalyzer/Tapestation

28. This is your pooled library ready for sequencing.

**NOTE 1: Library Quantification**

The final yield should be 10 μL of amplified library at 5 to 50 nM, with most of the fragments between 200 and 600 bp. The size distribution can be verified by running the library undiluted on an Agilent Bioanalyzer 2100 DNA High Sensitivity chip or equivalent, which will also give a rough reading of the concentration. The electropherogram may show evidence of overamplification: a secondary bump or especially wide smear of molecules that migrate more slowly than the rest, because they comprise complementary annealed adapters and noncomplementary, unannealable inserts. These libraries can still be sequenced, but the Bioanalyzer will report inaccurate molarities, and it is ideal to recalibrate the PCR cycles to the maximum number that does not produce this artifact. A small bump at about 85 bp is a normal byproduct of the TS-RT reaction and should not affect sequencing; a spike at about 160 bp indicates adapter dimers that will reduce the number of usable sequence reads, which are caused by too many PCR cycles.

**NOTE 2: Primers for Template-switching reverse transcription**

Use RNase-free HPLC purification for all. This reduces the complexity of the random bases, but that is less important than the purity of full-length molecules. The 2S primer may need to be ordered as an “RNA oligo” (e.g.IDT requires this) since it contains some ribonucleotides.

**First strand (1S) primer:**

/5Biosg/GT GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TV

**Second strand (2S) primer:**

/5Biosg/CT ACA CGA CGC TCT TCC GAT CTN NNN NrGrG rG

**Abbreviations (IDT codes)**

/5Biosg/: 5′ biotin

V: equimolar mix of A, C, G

N: equimolar mix of A, C, G, T

rG: riboguanosine (all other nucleosides are deoxy)

\*: phosphorothioate backbone

**NOTE 3: PCR primers for single-indexing**

These primers add a single i7 index (6 nt) to each library. Use HPLC purification for all. Up to 47 libraries may be combined in one pool for sequencing (46 with Illumina’s two-color chemistry on the NextSeq, MiniSeq, and NovaSeq). See e.g. Bio Scientific's NEXTflex™ DNA Barcodes for alternative index schemes with higher plexity; designing your own index sequences is inadvisable as they require empirical validation.

P5 universal: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG AT\*C\* T

P7 indexed: CAA GCA GAA GAC GGC ATA CGA GAT [i7] GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT\*C\* T

where [i7] is one of the following index sequences:

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **INDEX** | **SEQUENCE** | **INDEX** | **SEQUENCE** | **INDEX** | **SEQUENCE** | **INDEX** | **SEQUENCE** |
| **001** | CGTGAT | **013** | TTGACT | **025** | ATCAGT | **037** | ATTCCG |
| **002** | ACATCG | **014** | GGAACT | **026** | GCTCAT | **038** | AGCTAG |
| **003** | GCCTAA | **015** | TGACAT | **027** | AGGAAT | **039** | GTATAG |
| **004** | TGGTCA | **016** | GGACGG | **028** | CTTTTG | **040** | TCTGAG |
| **005** | CACTGT | **017** | CTCTAC | **029** | TAGTTG | **041** | GTCGTC |
| **006** | ATTGGC | **018** | GCGGAC | **030** | CCGGTG | **042** | CGATTA |
| **007** | GATCTG | **019** | TTTCAC | **031** | ATCGTG | **043** | GCTGTA |
| **008** | TCAAGT | **020** | GGCCAC | **032** | TGAGTG | **044** | ATTATA |
| **009** | CTGATC | **021** | CGAAAC | **033** | CGCCTG | **045** | GAATGA |
| **010** | AAGCTA | **022** | CGTACG | **034** | GCCATG | **046** | TCGGGA |
| **011** | GTAGCC | **023** | CCACTC | **035** | AAAATG | **047** | CTTCGA |
| **012** | TACAAG | **024** | GCTACC | **036** | TGTTGG | **048** | TGCCGA |

[FULL TABLE EXCEL TABLE](https://www.dropbox.com/s/x3nh5x80b7r2gk5/SMART3Seq_Single-indexing_PCR_primers.xlsx?dl=0)

* Indices 1-16, 18-23, 25, and 27 match the TruSeq LT system so you can use that setting for a sample sheet in the Illumina Experiment Manager or BaseSpace. The other indices require loading special files.
* Index 23 is G-rich in sequencing orientation and therefore produces low signal on the two-color chemistry of the NextSeq, MiniSeq, and NovaSeq; avoid using index 23 on these platforms.
* Index 41 differs from indices 11 and 31 by only 2 bases; avoid pooling 11+41 or 31+41.

**NOTE 4: Adjusting PCR Cycles**

The number of PCR cycles (C) needs to be adjusted as a function of multiplex level (N)and number of cells such as:

$$C= 19 -\frac{log\left(N\*total RNA \left(ng\right) in sample \right)}{log\left(1.9\right)} $$

RNA ng can be estimated from number of cells and quality of the sample. 1000 cells can yield 6 ng from frozen but as low as 1ng from FFPE. Round to the next integer number of cycles. Overamplification may lead to wasted sequencing capacity.

***Table 1: PCR cycle adjustment***

|  |  |
| --- | --- |
| **Number of cycles**  | **Samples in pool (N)** |
| **1** | **5** | **10** | **20** |
| **Cells** | **100** | 16 | 13 | 12 | 11 |
| **500** | 13 | 11 | 10 | 8 |
| **2000** | 11 | 8 | 7 | 6 |
| **10000** | 8 | 6 | 5 | 4 |