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Gene-expression profiling by Smart-3SEQ

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We use this protocol and it's working

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Abstract

This protocol combines aspects of 3SEQ (Beck et al., PLOS ONE 2010), Smart-seq2 (Picelli et al. Nat Methods 2013), and UMIs (Kivioja et al., Nat Methods 2011) into a streamlined method for making a sequencing library to measure the expression of polyadenylated RNAs. It is fast (3 hr), inexpensive (< \$10 / library), sensitive (tested successfully with 10 pg human total RNA, equivalent to one cell), and scalable (all steps performed in PCR tubes or microplates). It generates digital gene-expression data equivalent to SEQ's: one read per RNA molecule in the starting material, at the 3' end of the gene, which allows simpler quantification of transcript abundance at the expense of most information about splicing isoforms.

Materials

Equipment

- Adjustable-volume micropipets
- Programmable thermal cycler(s)
- Mini-centrifuge for PCR tubes or microplates, e.g. Ohaus #FC5306, Fisher #14-100-143
- Required only for individual library option: Magnetic separation block for 0.2 mL PCR tubes or microplates, e.g. V&P #772F4-1, #771LD-4CS
- Required only for pre-SPRI pooling option: Magnetic separation block for 1.5/2.0 mL microcentrifuge tubes, e.g. Thermo Fisher #12321D
- Recommended: separate workstations for pre-PCR and post-PCR steps, to avoid contamination

Consumables

- Low-retention 0.2 mL PCR tubes or microplates, e.g. Axygen Maxymum Recovery, Eppendorf LoBind
- Required only for pre-SPRI pooling option: Low-retention 1.5 or 2.0 mL microcentrifuge tubes, e.g. Eppendorf LoBind
- Required only for Arcturus LCM with HS caps: GeneAmp 0.5 mL PCR tubes, Thermo Fisher #N8010611
- Recommended: low-retention disposable pipet tips, e.g. Eppendorf Dualfilter T.I.P.S. LoRetention

Reagents

- SMARTScribe reverse transcriptase includes 5X reaction buffer and 20 mM DTT), Clontech #639536
- Nuclease-free dNTP mix, 10 mM each, e.g. Thermo Fisher #R0192
- Trimethylglycine (TMG; also called glycine betaine), 5 M solution, e.g. Sigma-Aldrich #B0300
- RNase inhibitor, 20X, e.g. Thermo Fisher #AM2694
- Magnesium chloride (MgCl₂), 80 mM solution (200 mM for FFPE LM), e.g. Sigma-Aldrich #63069
- HiFi HotStart ReadyMix, 2X, Kapa #KK2601
- DNA SPRI bead mix, e.g. Beckman Coulter #B23317 or homemade
- 80% ethanol in molecular biology-grade water, freshly prepared on the day of the procedure
- DNA storage buffer, e.g. TE, TE+Tween, Qiagen EB (pure water is not a buffer and not recommended)
- Oligonucleotide primers (see "Oligonucleotide primer designs (Illumina-specific)")
- Required only if using cells or tissue: Triton X-100, 0.5% (v/v) in molecular biology-grade water Required only for LCM FFPE tissue: Proteinase K, e.g. NEB #P8107S
Required only for LCM FFPE tissue: Proteinase K inhibitor, EMD Millipore #539470 (resuspend at 5 mM in DMSO)
- Optional to reduce cost for pre-SPRI pooling option: DNA SPRI bead binding buffer

Oligos

Note

Make sure to order NGS grade oligos. Otherwise the sequencing yield may be lower than expected.

- 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 TV
- Second strand (2S) primer: /5Biosg/CT ACA CGA CGC TCT TCC GAT CTN NNN NIGG rG
- Unique dual index oligos: Unique Dural Index Kit (96 reactions) , 10x Genomics, # 634752

Safety warnings

Experimental design considerations

Gene-expression profiling requires large sample sizes for powerful statistical analysis, like any other biological experiment. Because this protocol is rapid and inexpensive, it is not necessary to scale down your experiment to fit time or budget constraints; it is better to sequence 10 biological replicates at 50% depth than 5 replicates at 100% depth (see e.g. Liu et al., Bioinformatics 2014). As such, some volumes in this protocol may be impractical to pipet accurately for a single tube, and are intended to be prepared in a master mix with excess volume.

Plan the allocation of your index sequences before beginning. If possible, every library in your entire experiment should have a unique index, allowing you to sequence them all together in every lane/flowcell and avoid confounding by any technical variation on the sequencer, though this is not likely to be large.

On the other hand, if you are pooling a very small number of libraries, you must take care to balance the color usage in the sequencer. See Illumina's "TruSeq Library Prep Pooling Guide" for details.

This method is sensitive over at least five orders of magnitude of input amount; the only variable that needs to be changed is the number of PCR cycles. It is ideal to dilute your more concentrated samples so that all samples in the experiment start from the same amount of material. However, an accurate measurement of that amount is crucial; fluorometry (Qubit, RiboGreen, Bioanalyzer) is strongly recommended instead of spectrophotometry.

Before start

Pre-SPRI pooling option

The normal protocol makes a separate library from each RNA sample (Option 1: Individual libraries). However, if you are working in large batches, you can combine the libraries before the purification step and elute the pool into the same volume, concentrating the yield (Option 2: Pre-SPRI pooling). This saves money and time, and also reduces the number of PCR cycles required to generate enough material for QC and sequencing. It is crucial to pool low-input libraries that would require too many PCR cycles if processed separately. Every library in the pool must have a different index sequence, and this option may result in less balanced read counts from library to library than if you quantify them separately.



Procedure

1h 27m

- 1 Place the **RNase inhibitor** and **SMARTScribe reverse transcriptase** On ice
- 2 Thaw the frozen **TS-RT reagents** to Room temperature in your pre-PCR workstation.
- 3 Start **Program 1: Frag + TS-RT** in a designated amplification-free thermal cycler to pre-warm it. 45m

Program 1: Frag + TS-RT

80 °C 00:00:00 Hold (add tube containing RNA and Fragmentation Mix)

80 °C 00:05:00

Note

The duration of fragmentation step needs to be optimized depending on the input material, e.g. fragmented RNA from FFPE samples may only need 30s fragmentation or none at all.

42 °C 00:00:00 Hold (add TS-RT Mix to tube)

42 °C 00:30:00

70 °C 00:10:00

4 °C 00:00:00 Hold

- 4 To 1 µL total RNA sample in a low-retention PCR tube or microplate well, add 4.0 µL Fragmentation Mix and mix by pipetting:

2.0 µL SMARTScribe first-strand reaction buffer, 5X

1.0 µL nuclease-free dNTP mix, 10 mM each

0.5 µL 1S primer, 20 uM

 0.5 µL MgCl₂, 80 mM



- 5 When **Program 1** has reached the 80 °C Hold , place the tube in the thermal cycler and end the hold.
- 6 When the thermal cycler reaches the 42 °C Hold , remove and briefly centrifuge the tube, then add 5 µL **TS-RT Mix** and mix by pipetting:

2.0 µL	TMG, 5 M
1.0 µL	DTT, 20 mM
0.5 µL	RNase inhibitor, 20X
0.5 µL	2S primer, 20 uM
1.0 µL	SMARTScribe reverse transcriptase, 100 U/uL

- 7 Immediately return the tube to the thermal cycler and end the 42 °C Hold .
- 8 Thaw the frozen PCR reagents to room temperature.
- 9 Just before needed, start **Program 2: PCR** in a thermal cycler designated for PCR, using the appropriate number of cycles for your library (see Note:"Choosing the number of PCR cycles").

42m

Note

Compute the number of required PCR cycles based on the amount of total RNA input, with the formula

$$\text{cycles} = 19 - \log (N * \text{ng total RNA per sample}) / \log (1.9)$$

where N is the number of samples you will combine with the pre-SPRI pooling option, or N = 1 if you use the individual library option.

Program 2: PCR

98 °C 00:00:00 Hold (add tube containing TS-RT product and PCR reagents)

98 °C 00:00:45

Cycles:

98 °C 00:00:15



60 °C 00:30:00

72 °C 00:10:00

End Cycles

72 °C 00:01:00

4 °C 00:00:00 Hold

10 Once **Program 1** reaches the 4 °C Hold remove and briefly centrifuge the tube, then, still in your pre-PCR workstation, add 25 µL **PCR primer mix** with this library's unique combination of indexes, 0 µL each.

11 Add 12.5 µL **HiFi HotStart ReadyMix 2X**, 1 µL **Unique dual index oligos**, 1.5 µL RNase/DNase free water, and mix by pipetting.

12 When **Program 2** has reached the 98 °C Hold , place the tube in the thermal cycler and end the hold.

13 Continue with the cleanup protocol for either Option 1 or Option 2.

Option 1: Individual libraries

5m

14 When **Program 2** reaches the 4 °C Hold and the sample has cooled, briefly centrifuge the tube, add 55 µL **diluted SPRI bead mix** and mix very well:

25 µL molecular biology-grade water

30 µL SPRI bead mix

15 Incubate the tube 00:01:00 at Room temperature

1m

16 Place the tube on the magnet and wait for the beads to separate completely (00:02:00 on V&P 771LD).

2m

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

**Note**

Note: low-retention pipet tips are not necessary for steps 17-20.

- 18 Still on the magnet, add  200 μL freshly prepared **80% ethanol** to the tube and wait  00:00:30 30s
- 19 Repeat Step 18.
- 20 Remove and discard the supernatant. Use a smaller pipet to collect residual droplets.
- 21 Leave the tube open to air-dry  00:01:00 . Do not overdry the pellet or it will be difficult to resuspend. 1m
- 22 Remove the tube from the magnet, thoroughly resuspend the pellet in  10 μL **DNA Storage Buffer** and wait  00:00:30 30s
- 23 Return the tube to the magnet and allow the beads to separate completely.
- 24 Transfer the supernatant to a new tube. This is your sequencing-ready library.

Option 2: Pre-SPRI pooling**13m**

- 25 When **Program 2** reaches the  4 °C Hold and the samples have cooled, briefly centrifuge the tubes, then combine the samples into a single  1.5 mL (N $\leq$ 37 samples) or  2.0 mL (N $\leq$ 50) low-retention tube.
- 26 Add  15*N μL **SPRI bead mix** and mix very well.



27 Incubate the tube  00:05:00 at  Room temperature .

5m

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

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

Note

Note: low-retention pipet tips are not necessary for steps 29 and 33-36.

30 Remove the tube from the magnet and resuspend the pellet in  96 μ L Re-SPRI Mix (briefly centrifuge the tube if necessary to collect the pellet:

 60 μ L molecular biology-grade water

 36 μ L SPRI bead mix or bead binding buffer

31 Incubate the tube  00:05:00 at  Room temperature

5m

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

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

34 Still on the magnet, add  1 mL freshly prepared **80% ethanol** to the tube and wait  00:00:30 .

30s

35 Remove and discard the supernatant, then repeat the wash (step 34).

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



- 37 Leave the tube open to air-dry  00:02:00 . Do not overdry the pellet or it will be difficult to resuspend. 2m
- 38 Remove the tube from the magnet, thoroughly resuspend the pellet in  10 μ L **DNA Storage Buffer** (briefly centrifuge the tube if necessary to collect the pellet) and wait  00:00:30 . 30s
- 39 Return the tube to the magnet and allow the beads to separate completely.
- 40 Transfer the supernatant to a new tube. This is your sequencing-ready library pool.

Starting from fresh-frozen tissue on an Arcturus LCM MicroCap

20m

- 41 Pre-warm an incubator at  60 °C with the metal CapSure incubation block inside.

Note

Note: It may help to place an open container of water in the incubator for humidity.

- 42 Aliquot  10 μ L **LCM Lysis Mix** into an 0.2 mL low-retention PCR tube:
-  4 μ L SMARTScribe first-strand reaction buffer, 5X
 -  2 μ L nuclease-free dNTP mix, 10 mM each
 -  1 μ L 1S primer, 20 uM
 -  1 μ L MgCl₂, 80 mM
 -  1 μ L Triton X-100, 0.5% (v/v)
 -  1 μ L RNase inhibitor, 20X
- 43 Tightly seal the MicroCap into the tube, then flick it upside-down to cover the cap with the liquid.
- 44 Place the upside-down cap and tube in the pre-warmed incubation block.



45 Incubate  00:20:00 at  60 °C

20m

46 Pre-warm a designated amplification-free thermal cycler with **Program 1a: TS-RT Only**.

Program 1a: TS-RT Only

 42 °C  00:00:00 Hold (add TS-RT Mix to tube)

 42 °C  00:30:00

 70 °C  00:10:00

 4 °C  00:00:00 Hold

47 When the incubation is complete, briefly centrifuge the tube + cap to collect the lysate, then remove the cap. You can inspect it under a microscope later to verify complete lysis.

48 Add  10 µL **LCM TS-RT Mix** to the tube and mix by pipetting:

 4 µL TMG, 5 M

 2 µL DTT, 20 mM

 1 µL molecular biology-grade water

 1 µL 2S primer, 20 uM

 2 µL SMARTScribe reverse transcriptase, 100 U/uL

49 Place the tube in the thermal cycler and end the  42 °C Hold .The lid of the tube might not seal as well after the MicroCap has stretched it out, but the lid of the thermal cycler will hold it closed.

50 Continue with the normal protocol from step 47 (thaw the PCR reagents). Follow the special instructions for "Using damaged RNA". Double the volumes of the PCR primers and PCR master mix. In the SPRI cleanup, simply use 35 uL SPRI bead mix with no water (Option 1) or 35 x N uL (Option 2, first cleanup; keep the second cleanup the same).

**Note****Using damaged RNA**

If your RNA sample is already very fragmented (eg. from FFPE tissue), use an 0.7X SPRI ratio instead of 0.6X, i.e. use 35 μ L of SPRI bead mix to prepare the dilute SPRI bead mix and then use 60 L of that (individual library option), or $17.5 \times N$.L followed by 60 L water + 42 μ L SPRI mix (pre-SPRI pooling option). Expect a narrow distribution of smaller library molecules, and a lower percentage of alignable reads, which align very close to the end of each transcript.

Starting from FFPE tissue on an Arcturus LCM MicroCap

1h

- 51 Pre-warm an incubator at 60 °C with the metal CapSure incubation block inside.

Note

Note: It may help to place an open container of water in the incubator for humidity.

- 52 Aliquot 10 μ L **FFPE LCM Lysis Mix** into an 0.2 mL low-retention PCR tube:

4 μ L TMG, 5M

2 μ L nuclease-free dNTP mix, 10 mM each

1 μ L 1S primer, 20 μ M

1 μ L Triton X-100, 0.5% (v/v)

2 μ L Proteinase K, diluted to 0.125 g/ μ L in molecular biology-grade water

- 53 Tightly seal the MicroCap into the tube, then flick it upside-down to cover the cap with the liquid.

- 54 Place the upside-down cap and tube in the pre-warmed incubation block.

- 55 Incubate 01:00:00 at 60 °C

1h

- 56 Pre-warm a designated amplification-free thermal cycler with **Program 1a: TS-RT Only**.

**Program 1a: TS-RT Only**

- 42 °C 00:00:00 Hold (add TS-RT Mix to tube)
- 42 °C 00:30:00
- 70 °C 00:10:00
- 4 °C 00:00:00 Hold

57 When the incubation is complete, briefly centrifuge the tube + cap to collect the lysate, then remove the cap. You can inspect it under a microscope later to verify complete lysis.

58 Add 10 µL **FFPE LCM TS-RT Mix** to the lysate and mix by pipetting:

- 4 µL SMARTScribe first-strand reaction buffer, 5X
- 2 µL DTT, 20 mM
- 1 µL RNase inhibitor, 20X
- 0.4 µL 2S primer, 50 uM
- 0.4 µL MgCl₂, 200 mM
- 0.2 µL Proteinase K inhibitor, 5 mM
- 2 µL SMARTScribe reverse transcriptase, 100 U/ul

59 Place the tube in the thermal cycler and end the 42 °C Hold .The lid of the tube might not seal as well after the MicroCap has stretched it out, but the lid of the thermal cycler will hold it closed.

60 Continue with the normal protocol from step 57 (thaw the PCR reagents). Follow the special instructions for "Using damaged RNA". Double the volumes of the PCR primers and PCR master mix. In the SPRI cleanup, simply use 35 L SPRI bead mix with no water (Option 1) or 35 x N uL (Option 2, first cleanup; keep the second cleanup the same).

Note**Using damaged RNA**

If your RNA sample is already very fragmented (eg. from FFPE tissue), use an 0.7X SPRI ratio instead of 0.6X, i.e. use 35 uL of SPRI bead mix to prepare the dilute SPRI bead mix and then use 60 L of that (individual library option), or 17.5 x N .L followed by 60 L water + 42 uL SPRI mix (pre-SPRI pooling option). Expect a narrow distribution of smaller library molecules, and a lower percentage of alignable reads, which align very close to the end of each transcript.