Protocol

Protocol for RNA-seq library preparation starting from a rare muscle stem cell population or a limited number of mouse embryonic stem cells

It remains challenging to generate reproducible, high-quality cDNA libraries from RNA derived from rare cell populations. Here, we describe a protocol for high-throughput RNA-seq library preparation, including isolation of 200 skeletal muscle stem cells from mouse tibialis anterior muscle by fluorescence-activated cell sorting and cDNA preparation. We also describe RNA extraction and cDNA preparation from differentiating mouse embryonic stem cells.

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Highlights

- FACS isolation of 200 muscle stem cells (MuSCs) from one mouse tibialis anterior muscle
- cDNA library construction for deep sequencing by direct lysis of 200 MuSCs
- cDNA library construction for deep sequencing from 5,000–10,000 embryonic stem cells
Protocol

Protocol for RNA-seq library preparation starting from a rare muscle stem cell population or a limited number of mouse embryonic stem cells

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SUMMARY

It remains challenging to generate reproducible, high-quality cDNA libraries from RNA derived from rare cell populations. Here, we describe a protocol for high-throughput RNA-seq library preparation, including isolation of 200 skeletal muscle stem cells from mouse tibialis anterior muscle by fluorescence-activated cell sorting and cDNA preparation. We also describe RNA extraction and cDNA preparation from differentiating mouse embryonic stem cells. For complete details on the use and execution of this protocol, please refer to Juan et al. (2016) and Garcia-Prat et al. (2016).

BEFORE YOU BEGIN

The main purpose of this protocol is to permit the isolation of RNA from a low number of cells to perform RNA-seq. Before starting the protocol, it is advisable to wipe all working surfaces and instruments with RNAseZAP RNase Decontamination Solution.

⚠️ CRITICAL: Unless otherwise specified, all reagents and materials used in the RNA extraction section and below are certified as DNase- and RNase-free. Collect cells in Trizol.

Note: All reagents should be equilibrated to ~25°C before starting RNA extraction.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| PE-Cy7-conjugated anti-CD45 | BioLegend | 103114     |
| PE-Cy7-conjugated anti-Sca-1   | BioLegend | 108114     |
| PE-Cy7-conjugated anti-CD31   | BioLegend | 102418     |

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| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| PE-conjugated anti-ITGA7 | AbLab  | AB10STMW215 |
| Alexa Fluor 647-conjugated anti-CD34 | BD Pharmigen | 560230 |

Critical commercial assays

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| RNase AWAY Decontamination Reagent | Thermo Fisher Scientific | Cat# 10228011 |
| TRIzol reagent | Thermo Fisher Scientific | Cat# 15596018 |
| Absolute ethanol | Fisher Scientific | Cat# BP2818100 |
| GlycoBlue Coprecipitant | Applied Biosystems | AM9515 |
| AMPure XP beads | Beckman Coulter | Cat# A63881 |
| Nuclease-free water | Invitrogen | Cat# AM9937 |
| Qubit dsDNA HS kit | Thermo Fisher Scientific | Cat# Q32851 |
| Invitrogen Qubit Fluorometer | Thermo Fisher Scientific | Cat# Q33240 |
| Agilent High Sensitivity DNA Kit | Agilent Technologies | Cat# 5067-4626 |
| Quant-iT PicoGreen dsDNA Assay Kit | Thermo Fisher Scientific | P11496 |
| High Sensitivity RNA ScreenTape | Agilent Technologies | 5067-5579 |
| High Sensitivity RNA ScreenTape Sample Buffer | Agilent Technologies | 5067-5580 |
| High Sensitivity RNA ScreenTape Ladder | Agilent Technologies | 5067-5581 |
| High Sensitivity DNA ScreenTape | Agilent Technologies | 5067-5584 |
| High Sensitivity D1000 Sample Buffer | Agilent Technologies | 5067-5603 |
| High Sensitivity D1000 Ladder | Agilent Technologies | 5067-5587 |
| SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing | Takara | 634891 |
| NEBNext Poly(A) mRNA Magnetic Isolation Module | NEB | E7490 |
| NEBNext Ultra II RNA Library Prep Kit for Illumina | NEB | E7770 |
| DAPI | Thermo Fisher | Cat# D3571 |
| PBS | Sigma-Aldrich | D8537 |
| FBS | Gibco | Cat# 1027-106 |
| Ham’s F10 medium | Roche | #177246 |
| Dulbecco’s modified Eagle medium | Biowest | L0101-500 |
| Dispase | Gibco | #17105-041 |
| Liberase | Roche | #177246 |
| BD Pharm Lyse | BD | #55899 |

Experimental models: organisms/strains

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| E14 mouse embryonic stem cells | ATCC CRL-1821 | ATCC |
| C57BL/6J mice | The Jackson Laboratory | RRID:IMSR_JAX:000664 |

Other

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| TipOne filter tip 10 μl | USA Scientific | Cat# 1120-3810 |
| TipOne filter tip 20 μl | USA Scientific | Cat# 1120-1810 |
| TipOne filter tip 200 μl | USA Scientific | Cat# 1120-8810 |
| TipOne filter tip 1,000 μl | USA Scientific | Cat# 1120-1830 |
| PCR tube | USA Scientific | Cat# 14024700 |
| DNA LoBind tubes (1.5 mL) | Eppendorf | Cat# Z665648 |
| Falcon tube (50 mL) | Coming | Cat# CLS430290-500EA |
| Falcon tube (15 mL) | Coming | Cat# CLS430053-500EA |
| 100 μm Strainer filters | Coming | Cat# 352360 |
| 70 μm Strainer filters | Coming | Cat# 352350 |
| 40 μm Strainer filters | Coming | Cat# 431750 |
| SPRIME Phase Lock Heavy Gel | Quantabio | 2302830 |
| Minifuge for PCR TubeStrips | Major supplier | N/A |
| Thermocycler | Major supplier | N/A |
| Refrigerated centrifuge | Eppendorf | Cat# 5430R |
| Centrifuge with a cooling system for 15–50 mL conical tubes | Beckman Coulter | N/A |
| Shaking water bath | N/A | N/A |
| Agilent 4200 TapeStation | Agilent Technologies | Cat# G2991AA |
| Biosafety cabinet | N/A | N/A |

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MATERIALS AND EQUIPMENT

Alternatives: This protocol requires the use of a Qubit Fluorometer (Thermo Fisher Scientific) to accurately measure the concentration of total RNA recovered from RNA extraction. If a Qubit Fluorometer is not available, a PicoGreen staining may be employed to evaluate RNA concentration using a plate reader. Alternatively, this step can be omitted and the RNA analyzed with a Fragment Analyzer Systems (Agilent) to assess both quality and quantity. We strongly suggest analyzing the RNA quality with a Fragment Analyzer Systems before proceeding further.

Pause point: Extracted and purified RNA samples can be stored at −80°C for a maximum of two weeks but we recommend continuing the protocol up to the conversion of RNA into cDNA. Earlier stopping points can negatively affect the final library complexity.

STEP-BY-STEP METHOD DETAILS

Muscle dissection, antibody staining, and flow activated cell sorting (FACS)

© Timing: ~6 h

1. 200 MuSCs cells are isolated from skeletal muscle tissue following tissue digestion and FACS protocols.
   a. The starting material consists of at least one muscle, such as the tibialis anterior (TA), from one mouse, and can be scaled up to additional muscles and biological replicates. Digestion of one TA muscle is sufficient to obtain 200 MuSCs after the completion of this protocol; however, other hindlimb and forelimb muscles can be included, such as gastrocnemius, soleus, quadriceps, extensor digitorum longus (EDL) and triceps, to isolate higher numbers of MuSCs.
   b. In order to obtain an unicellular suspension of skeletal muscle ready for FACS, the muscle tissue is isolated from previously euthanized mice and digested following the protocol described below. The following steps should be performed in a tissue culture hood to avoid contamination.
   c. Dissect muscle tissue with small scissors and collect it in cold Dulbecco’s Modified Eagle Medium (DMEM) with 1% penicillin/streptomycin (P/S) into 50 mL Falcon tubes. Decant muscle into a petri dish and remove DMEM 1% P/S completely. Mince the muscle using small scissors and razor blades until small pieces are achieved and place them into new 50 mL Falcon tubes with the digestion mix, composed by Ham’s F10 medium containing 5 mg/mL of liberase (Roche, #177246), 0.4 mM CaCl₂, 5 mM MgCl₂ and 0.3% dispase (Gibco, #17105-041). We recommend using 5 mL of digestion buffer per 1 g of initial muscle tissue material, which can be increased up to 10 mL for larger samples (1–2 g) or more fibrotic samples (for example, muscle tissue from very old mice).
   d. Incubate the samples in a shaking water bath at 37°C for 1–2 h. Centrifuge the cells at 50 g for 10 min at 4°C, collect the supernatant and discard the pellet.

Optional: wash the pellet and collect the supernatant again to improve cells’ recovery.
   e. Consecutively filter the supernatant through 100 µm and 70 µm strainer filters into new 50 mL Falcon tubes. Following a centrifuging step at 670 g for 15 min at 4°C, collect the supernatant and discard the pellet.

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cell culture incubator | N/A | N/A |
| Dissection tools | N/A | N/A |
| BD FACS Aria | BD Biosciences | N/A |
| Illumina NovaSeq 6000 | Illumina | Cat# 20012850 |
| Illumina NextSeq 550 | Illumina | Cat# SSY-415-1002 |
supernatant, gently resuspend the cells in 2 mL of lysis buffer (BD Pharm Lyse, #555899) to lyse red blood cells following the manufacturer’s recommendations and incubate for 10 min on ice. Next, add 40 mL of cold FACS buffer, composed by PBS supplemented with 2.5% fetal bovine serum (FBS), filter the sample through a 40 μm strainer filter into new 50 mL Falcon tube and count the cells. Centrifuge at 670 g for 15 min at 4°C, remove the supernatant and resuspend the cells at 1×10^6 cells/mL concentration in the FACS buffer.

g. Stain the sample with PE-Cy7-conjugated anti-CD45, PE-Cy7-conjugated anti-Sca-1, PE-Cy7-conjugated anti-CD31, Alexa Fluor 647-conjugated anti-CD34 and PE-conjugated anti-ITGA7 antibodies for 30 min on ice, protected from light (see Key Resources Table for details). Finally, wash the cells with 40 mL of cold PBS, centrifuge at 670 g for 15 min at 4°C, remove the supernatant and resuspend the sample in 0.5 mL of cold PBS (add more PBS if digestion yields are >1M of cells). Filter through a test tube with cell strainer cap to eliminate cell aggregates and stain with DAPI (final concentration 1 μg/mL) 5 min prior to FACS (Sousa-Victor et al., 2014) (Garcia-Prat et al., 2016).

h. Based on the relative expression of different markers (in this case CD34 and ITGA7), collect 200 cells of distinct MuSCs populations from the same sample (Garcia-Prat et al., 2020). For this purpose, we employ a FACS Aria II sorter (BD) with 4-way purity precision mode that allows simultaneous separation of a maximum of 4 populations. Employ cell granularity (side scatter, SSC-A), cell size (forward scatter, FSC-A) and DAPI staining to exclude cell aggregates, debris, and dead cells. PE-Cy7+ events (corresponding to CD45+, Sca-1+ and CD31+ cells) are used for lineage-negative selection and Alexa Fluor 647+ and PE+ (which identify CD34+ and ITGA7+ populations respectively) for double positive staining of MuSCs (Figure 1). Sort MuSCs directly into PCR tubes pre-filled with 10 μl of Reaction buffer to avoid unnecessary transferring steps and loss of material.
Reverse transcription of RNA derived from FACS-sorted MuSCs

© Timing: ~3 h

2. We employ the Takara “SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing” (Cat. Nos. 634888, 634889, 634890, 634891, 634892, 634893, and 634894) to generate full-length cDNAs directly from 200 FACS-isolated MuSCs. The protocol allows direct cell lysis and retrotranscription without prior RNA extraction.

Note: The following steps are conducted as described in the TAKARA protocol accompanying the kits with minor changes listed below (Figure 2). This is the link to the detailed protocol:

https://www.takarabio.com/a/114896

3. Prepare a stock solution of 10× Reaction Buffer by mixing 19 µL of the 10× Lysis Buffer with 1 µL of the RNase inhibitor.

4. Prepare a solution of 1× Reaction Buffer by mixing nuclease-free water and the 10× Reaction Buffer. Place 10 µL of the 1× Reaction Buffer directly into an individual 0.2 mL RNase-free PCR tube.

Note: We estimated that the amount of FACS flow buffer coming from droplets with 200 MuSCs leads to approximately 0.4–0.5 µl with 4-way purity precision mode and 85 µm nozzle size, and therefore, adjusted the 1× Reaction Buffer volume accordingly. We recommend running prior tests to determine volume increase resulting from FACS flow buffer to avoid Reaction Buffer dilution. This volume depends on the sorting machine and parameters employed, such as nozzle size and precision mode.

5. Resuspend stained cells in PBS and directly sort 200 MuSCs into an RNase-free PCR tube with 9 µL of 1× Reaction Buffer.

Note: Avoid using FBS or other serum, which are usually placed into FACS buffer, during sorting since it may interfere with the following RNA transcription steps.

6. Incubate the cell:reaction buffer mix at ~25°C for 5 min.

7. Place the samples on ice and add 2 µl of 3′ SMART-Seq CDS Primer II A. Mix by gentle vortexing and then spin the tube(s) briefly to collect the contents at the bottom of the tube.

Note: Bring the final volume to 12.5 µl by adding additional Nuclease-Free Water if necessary

8. Incubate the tubes at 72°C in a preheated, hot-lid thermal cycler for 3 min. Immediately place the samples on ice for 2 min.
**Note:** Prepare the First Strand Master Mix

(See Buffer preparation instructions in the manufacturer’s protocol while the samples are incubating. https://www.takarabio.com/a/114896)

9. Preheat the thermal cycler to 42°C.
10. Add 2 μL per reaction, plus 10%, of the SMARTScribe Reverse Transcriptase to the Master Mix.

**Note:** Add the reverse transcriptase to the Master Mix just before use, making sure to gently mix the reverse transcriptase tube without vortexing before adding it.

**Note:** Mix the Master Mix well by gently vortexing and then spin the tube(s) briefly in a mini-centrifuge to collect the contents at the bottom of the tube.

11. Add 7.5 μl of the Master Mix to each reaction tube. Mix the contents of the tubes by gently pipetting and spin them briefly to collect the contents at the bottom of the tubes.
12. Place the tubes in a thermal cycler with a heated lid, preheated to 42°C, and run the following program:

| Temperature | Time |
|-------------|------|
| 42°C        | 90 min |
| 70°C        | 10 min |
| 4°C         | Forever |

**Pause point:** The tubes can be stored at 4°C for 12–18 h.

13. Thaw all the reagents needed for PCR (except the enzyme) on ice. Gently vortex each reagent tube to mix and spin down briefly. Store on ice.
14. Prepare PCR Master Mix combining the following reagents in the order shown:
15. Add 30 μl of PCR Master Mix to each tube containing 20 μL of first-strand cDNA product from step 11.
16. Place the samples in a preheated thermal cycler with a heated lid and run the following program. Refer to manufacture instructions to determine the optimal number of cycles (N). https://www.takarabio.com/a/114896

| PCR Cycling Conditions | Temperature | Time | Cycles |
|------------------------|-------------|------|--------|
| Initial Denaturation   | 98°C        | 1 min| 1      |
| Denaturation           | 98°C        | 10 s | N cycles |
| Annealing              | 65°C        | 3 min|        |
| Final extension        | 72°C        | 10 min|      |
| Hold                   | 4°C         | Forever| 1      |

**Pause point:** The tubes may be stored at 4°C for 12–18 h.

17. Purify the amplified cDNA using the Agencourt AMPure XP Kit (Beckman Coulter, Cat# A63881) according to Takara instructions https://www.takarabio.com/a/114896.
RNA extraction by TRIzol

© Timing: ~2 days

As an alternative to the Takara SmarterSeq v4 kit, when direct lysis of cells is not possible, we suggest extracting RNA by collecting cells directly in a 1.5 mL Eppendorf tube with 250 μL of Trizol. The following protocol has been optimized using mouse embryonic stem (E14 ESCs) (Juan et al., 2016).

The following protocol allows recovering of sufficient RNA to generate a library from 5,000 to 10,000 cells (Figure 3A).

Note: All the reagents used are certified as DNase and RNase free.
**Critical:** Trizol is a phenol-based reagent and must always be used in a biochemical hood. Eyes and skin exposure should be avoided. Follow the safety data sheet when handling Trizol.

**Pause point:** Cells can be stored in Trizol at ~80°C for long-term storage.

18. Thaw Trizol samples at ~25°C, if frozen.
19. Let samples stand at ~25°C for 5–10 min.
20. During this time, pre-spin the 5PRIME Phase Lock Heavy Gel tubes (QuantaBio, Cat # 2302830) at 1,600 g for 45 sec to collect gel on tube bottoms.

*Note:* 5PRIME Phase Lock Heavy Gel tubes facilitate the recovery of the aqueous phase and minimize potential carry-over from the phenol-chloroform phase. Alternatively, the two phases can be separated following the Trizol manufacturer's protocol.

21. Add cells already lysed in Trizol to the 5PRIME Phase Lock Heavy Gel tubes and incubate for 5 min at ~25°C.
22. Examine phasing.

*Note:* Clear, aqueous phase should be entirely at the top of the 5PRIME Phase Lock Heavy Gel. The phenol-chloroform phase and cloudy interphase should be below 5PRIME Phase Lock Gel's layer.

23. Add 100 μl Chloroform
24. Manually invert the tubes 5 times.

*Note:* Do not vortex.

25. Incubate at ~25°C for 10 min.
26. Centrifuge at 12,000 g at 4°C, for 15 min.

*Note:* Examine the Eppendorf tube. A transparent, aqueous phase should be located at the top of the 5PRIME Phase Lock Heavy Gel. The phenol-chloroform phase and a cloudy interphase should both be below the Phase Lock Gel layer.

27. Centrifuge at 12,000 g at 4°C for 15 min.
28. Recover and transfer the rest of the aqueous phase with a 20 μl pipette.

**Critical:** Samples with a high content of fat, protein, polysaccharides, or extracellular material may require additional clean-up steps. ([https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0016385_TRIzol_Reagent_DNA_Isol_UG.pdf&title=VXNlciBHDwkZSATlFRSSXpwbCBSZWFnZW50IC0gRXhwZXJpbWVudGFsIHByb3RvY29sIGZvciBETkEgaXNvbGF0aW9u](https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0016385_TRIzol_Reagent_DNA_Isol_UG.pdf&title=VXNlciBHDwkZSATlFRSSXpwbCBSZWFnZW50IC0gRXhwZXJpbWVudGFsIHByb3RvY29sIGZvciBETkEgaXNvbGF0aW9u)).

29. Add 10 μg of molecular grade Glycogen (or GlycoBlue).
30. Add 250 μl of molecular grade isopropanol.
31. Invert tube 10 times to mix (or vortex on a ‘low’ setting for 15 sec)
32. Let samples precipitate at ~20°C for 12–18 h.

*Note:* Precipitation can be shortened to 60–90 min, however, for maximum recovery yield we recommend performing this step for 12–18 h.
33. Centrifuge at 12,000 g, 4°C, for 30–45 min
34. Gently discard the supernatant leaving approximately 10–15 μl behind.

   **Note:** The RNA pellet may detach from the tube and float.

   **Note:** When starting with less than 100,000 cells a pellet cannot be visualized by eye. We suggest removing the tubes from the centrifuge very slowly and identifying the side of the tube where the pellet is located after centrifugation (Figure 3B).

35. Add 500 μl of freshly prepared 75% EtOH, mix 10 times by inversion.
36. Centrifuge at 7,500 g, 4°C for 5 min.
37. Gently discard the supernatant leaving approximately 10–15 μl behind without disturbing the pellet.

   **Note 1:** The RNA pellet may detach from the tube and float.

   **Note 2:** When starting with less than 100,000 cells a pellet cannot be seen by eye. We suggest removing the tubes from the centrifuge very slowly and identifying the side of the tube where the pellet is located after centrifugation.

38. Slowly remove most of the residual EtOH with a p10 pipette leaving 2–5 μl of EtOH (Figure 3C).
39. Air dry the RNA pellet for about 15–20 min.
40. Re-suspend the RNA pellet in 15 μL of RNAse/DNAse free water. Keep the resuspended RNA on ice and immediately proceed to the next step.

   △ CRITICAL: Remove as much ethanol as possible using a 10 μL pipette before air-drying the pellet for approximately 5 min. Do not over-dry the pellet. Over-drying will decrease RNA solubility. Air-dry the pellet in a clean, RNase-free location.

41. Proceed with DNase treatment using a commercially available kit. We used the Turbo-DNA free kit from Invitrogen, following manufacturer instructions with no modifications (http://tools.thermofisher.com/content/sfs/manuals/cms_055740.pdf).
42. Perform polyadenylation enrichment, cDNA conversion, and fragmentation RNA quality assessment and quantification.

### RNA quantification and quality control

**Timing:** ~30 min

43. Quantify the amount of total RNA recovered using a Qubit instrument (See Materials and Equipment section for alternatives)

   **Note:** RNA at this stage can also be quantified by Nanodrop but the limited sensitivity of this instrument leads to imprecise quantification for low amounts of RNA.

   Analyze the quality of the RNA using a Fragment Analyzer.

   **Note:** Degraded RNA (RNA integrity number, RIN <7) will decrease the efficiency of cDNA conversion and library preparation.

### cDNA conversion

**Timing:** ~2 h
Following RNA quantification, 10–250 μg of RNA is enriched for mRNAs by polyadenylation selection, converted to cDNA, and fragmented.

The steps are performed using NEB buffers and enzymes. Currently, all the required reagents are part of the “NEBNext Poly(A) mRNA Magnetic Isolation Module” (E7490) and “NEBNext Ultra II RNA Library Prep Kit for Illumina” (E7770, E7775).

**Note:** The following steps are conducted exactly as described in the NEB protocol accompanying the kits. This is the link to the extended protocol:

https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr

The same steps described in the protocol are reported here:

44. Prepare First-Strand Reaction Buffer and Random Primer Mix according to manufacturer instructions in a nuclease-free microcentrifuge tube. ([https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr](https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr)).

**Note:** Keep the mix on ice until mRNA is purified.

45. Dilute the total RNA with nuclease-free water to a final volume of 50 μl in a nuclease-free 0.2 mL PCR tube.

46. Prepare and wash oligo-dT beads according to manufacturer instructions ([https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr](https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr)).

47. Add 50 μl beads to each RNA sample. Mix thoroughly by pipetting up and down six times.

48. Incubate at 25°C for 5 min.

49. Move the samples onto a magnetic rack to separate the beads and discard the supernatant.

50. Wash the beads twice according to manufacturer’s instructions ([https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr](https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-mrna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-rr)).

51. Add 50 μl of Tris buffer (provided in NEB #E7490 kit) to each tube. Gently pipette up and down 6 times.

52. Place the tube on the thermocycler with the heated lid set at ≥ 90°C and run the following program:

| PCR Conditions | Temperature | Time |
|----------------|-------------|------|
|                | 80°C        | 2 min|
|                | 25°C        | Forever|

**Note:** Remove the tube from the thermocycler only when the temperature reaches 25°C.

53. Add 50 μl of RNA Binding Buffer (2×) to the samples. Mix by gently pipetting up and down six times.

54. Incubate the tube at ~25°C for 5 min.

55. Place the tube on the magnetic rack at ~25°C until the solution is clear.

56. Remove and discard the supernatant from the tube.
**Note:** Do not disturb the beads.

57. Remove the tube from the magnetic rack.
58. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times.
59. Spin down the tube briefly to collect the liquid from the wall and lid of the tube.

**Note:** It is important to spin down the tube to prevent carryover of the Wash Buffer in subsequent steps.

60. Place the tube on the magnet at ~25°C until the solution is clear (~2 min).
61. Remove and discard all of the supernatants.

**Note:** It is important to completely remove the supernatant to successfully fragment the mRNA in the subsequent steps.

62. Remove the tube from the magnetic rack.
63. Add 11.5 µl of the First-Strand Synthesis Reaction Buffer and Random Primer Mix, pipette six times to resuspend the beads.
64. Incubate the sample in a thermal cycler with the heated lid set at 105°C and run the following program:

| Temperature | Time     |
|-------------|----------|
| 95°C        | 15 min   |
| 4°C         | Forever  |

**Note:** Immediately transfer the tube on ice for 1 min as soon as it is cool enough to handle (~65°C)

65. Collect the fragmented mRNA by transferring 10 µL of the supernatant to a nuclease-free 0.2 mL PCR tube.

**Note:** If the volume recovered is less than 10 µl bring the volume up to 10 µL by adding the First-Strand Synthesis Reaction Buffer and Random Primer Mix.

66. Place the tube on ice and proceed directly to First Strand cDNA Synthesis.
67. Add to the samples 8 µL of Nuclease-free Water and 2 µL of NEBNext First-Strand Synthesis Enzyme Mix and pipette up and down 15 times.
68. Put the samples in a preheated thermocycler with the heated lid set at ≥ 80°C and run the following program:

| Temperature | Time     |
|-------------|----------|
| 25°C        | 10 min   |
| 42°C        | 15 min   |
| 70°C        | 15 min   |
| 4°C         | Forever  |
Immediately perform Second Strand cDNA Synthesis.

**Note:** Assemble the second strand cDNA synthesis reaction on ice.

69. Add to the samples 8 μL of “NEBNext Second Strand Synthesis Reaction Buffer” and 48 μL of Nuclease-free Water. Mix by pipetting the reaction up and down 15 times.

70. Put the samples in a thermocycler set for 1 h at 16°C14°C with the heated lid at ≤ 40°C (or off).

71. Purify the obtained cDNAs using SPRIselect Beads according to NEB instructions (https://international.neb.com/protocols/2017/03/04/protocol-for-use-with-nebnext-poly-a-rna-magnetic-isolation-module-e7490-and-nebnext-ultra-ii-r).

**Library preparation**

**@ Timing:** ~3 h

72. cDNAs synthesized following “SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing” are quantified by Qubit or Picogreen and further converted into a library using the “NEBNext Ultra II DNA library prep for Illumina” kit (New England Biolabs, E7645L) following manufacturer’s instructions (https://www.neb.com/-/media/nebus/files/manuals/manuale7645.pdf?rev=8b4c7f67ccca4b4a805ceed982ef49b6).

Adapters are diluted 25 times and 9 cycles of PCR are used for libraries amplification.

mRNAs enriched by polyadenylation selection (step 42, RNA Extraction by Trizol) are converted to cDNA, and fragmented using NEB buffers and enzymes included in the “NEBNext Ultra II RNA Library Prep Kit for Illumina” (E7770, E7775).

The final libraries are quantified by Picogreen (Invitrogen) and size distribution is determined on a Fragment Analyzer.

**Sequencing**

**@ Timing:** ~18–20 h

73. Libraries are equalized to the same molarity and pooled for sequencing on a NextSeq500 (Illumina) or NovaSeq3000 (Illumina). Following ENCODE’s recommendations, samples are sequenced at a depth of ~40 M reads/sample. Libraries are also compatible with sequencing on other Illumina instruments with minor adjustments during library preparation as described in the manufactured protocols (https://www.neb.com/-/media/nebus/files/manuals/manuale7645.pdf?rev=8b4c7f67ccca4b4a805ceed982ef49b6).

**EXPECTED OUTCOMES**

**SMART-seq v4 Ultra Low Input RNA Kit for Sequencing**

1 μL of the final cDNA should be validated using a Fragment Analyzer. See the Agilent High Sensitivity DNA Kit User Manual for instructions (https://www.agilent.com/cs/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf).

Successful cDNA synthesis and amplification should show a distinct peak spanning 400 bp to 10,000 bp, peaked at ~2,500 bp.

The expected yield is 3.4–17 ng of cDNA (depending on the cell type and initial cell number).
RNA extraction by Trizol
The quality of the extracted RNA should be assessed by Fragment Analyzer. Ideally, the analysis should show a very small amount of RNA degradation, an RNA integrity number, RIN \( \geq 7 \), and the absence of genomic DNA contamination.

The amount of total RNA recovered correlates with the number of cells processed and varies for different cell types.

Library preparation
Libraries should have a total yield of 10–100 ng of cDNA with a size distribution centered on \(~300 \text{ bp}\).

LIMITATIONS
FACS
FACS analysis does not allow to separate more than 4 populations from one sample at the same time. Additionally, prolonged sorting duration resulting from the low frequency of the cell population of interest leads to high mortality of sorted cells and negatively impacts RNA quality. Efforts should be made to reduce the sorting time.

cDNA Libraries
The quality of the initial RNA is the prime determinant for generation of an optimal cDNA library. Moreover, first-strand cDNA synthesis using Takara Smarter Seq v4 requires isolation of RNA or cells in a maximum volume of 9.5 \( \mu l \). Therefore, special care should be taken to limit volume variations and plastic retention of both RNA and cDNA.

Cell fixation and cultured cells
The protocol has not been tested for MuSCs that have been fixed in paraformaldehyde \textit{in vitro} or \textit{in vivo} prior to isolation. Finally, the procedures described here to generate libraries for both FACS-isolated MuSCs and mESCs have not been tested on cultured primary cells or cell lines. However, we anticipate that they will be adaptable for cultured cells.

TROUBLESHOOTING
Problem 1
Poor quality or insufficient cDNA yield (step 71).

Potential solution
The quality and quantity of the starting material is crucial when working with rare cell populations or low starting material. Exclude adipose tissue (white fat), nerves and tendons during dissection since they may impair the digestion efficiency, obstruct cell strainers during sample filtration steps and introduce cross-contamination with cell types from other close-by tissues.

Problem 2
Low MuSCs Yield (step 1d).

Potential solution
Digestion time can be prolonged to obtain higher cell yields, especially in the case of muscle tissue obtained from very old animals. However, sustained digestion can also lead to higher cell mortality rates, therefore, we do not recommend exceeding 2 h of digestion.

It is important to dispose of dead cells and debris as much as possible. During sample preparation, some cells lyse and release DNA, which leads to aggregate formation and hampers the sorting. Treatment with DNase prior to cell sorting helps to overcome this problem.
Problem 3
Low purity of FACS-sorted MuSCs (step 1f).

Potential solution
When working with very low number of cells, high purity of the sorted population should be achieved. To do so, use all necessary controls for FACS panel elaboration prior to sample sorting, including:
- Negative (unstained) controls: needed for lasers’ intensity set up and determining the sample autofluorescence.
- Single stained controls: individual staining with each antibody or dye, needed for compensation of false signal resulting from spectral overlap between two fluorochromes.
- Fluorescence minus one (FMO) controls: staining with all antibodies with an exception of one, which are needed to properly discriminate among the cell populations.

Enrichment of the sample with the population of interest by magnetic bead-based negative selection should be considered to reduce sorting duration.

Problem 4
RNA degradation (step 42).

Potential solution
While handling a low amount of RNA, it is important to maintain an RNase-free environment to avoid RNA degradation. We recommend working in a designated area with designated pipettes cleaned with RNAse ZAP decontaminant solution.

Problem 5
Insufficient RNA or cDNA (steps 42 and 71).

Potential solution
To minimize the loss of material, we suggest using low-binding PCR tubes and low retention tips for sorting, RNA extraction, cDNA retro-transcription, and library preparation.

Problem 6
Insufficient cDNA (steps 17 and 71).

Potential solution
Under- or over-drying the pellet during cDNA purification with AMPure XP magnetic beads may affect cDNA recovery and final yield. Under-dried pellet appears translucid, the residual ethanol may interfere with the final yield. Over-dried pellet will present cracks. It will take longer than 2 min to rehydrate, and final yield will again be affected.

It is advisable to carefully look at the pellet and adjust the drying time according with the appearance of the pellet.

Problem 7
Insufficient cDNA (step 16).

Potential solution
When possible, the optimal number of PCR cycles required for each experiment should be empirically determined in a parallel preparation. We recommend using the lowest PCR cycle number that generates enough material for library preparation.
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vittorio Sartorelli sartorev@mail.nih.gov

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any novel data or codes that are not presented in this published article directly.

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AUTHOR CONTRIBUTIONS
S.D. and A.H.J. developed RNA extraction and library preparation protocols. V.M. and L.G.-P. developed FACS isolation protocol. V.S and P.M.-C. developed and supervised the projects with input from all the authors. All the authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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