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sc-rDSeq: Droplet-based single-cell full-length total RNA-seq method

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Abstract

This protocol describes sc-rDSeq, a scalable, droplet-based method for full-length, strand-specific total RNA sequencing at single-cell resolution. The protocol uses a refined set of 220 ribosomal-depleted sequences (rDS) primers that selectively exclude ribosomal RNA during initial reverse transcription, enabling capture of both polyadenylated and non-polyadenylated RNAs such as histone RNAs, non-coding RNAs, and enhancer RNAs, without requiring costly post-amplification depletion steps. This method is useful for researchers who would like to detect not only gene expression variations, but also alternative splicing events and single nucleotide variations in complex heterogenous cellular systems, providing a more complete view of cellular heterogeneity and regulatory programs that remain invisible to conventional polyadenylated-only sequencing approaches. Compared with existing full-length protocols, which are often limited by high reagent costs or reliance on complex multistep microfluidics, sc-rDSeq provides a simpler, single-step microfluidic workflow compatible with standard inDrops platforms, which may reduce experimental complexity and cost relative to existing full-length total-RNA methods. A key improvement is the 10-fold increase in unique molecular identifiers per cell relative to 3′ end-based methods, at a reported reagent cost of approximately $0.08 per cell, making deep total transcriptome analysis more accessible. The protocol includes three major parts: sc-rDSeq barcode synthesis, single-cell co-encapsulation, and library construction.

Keywords Single-cell total RNA-seq, Full transcriptomics, Drop-based microfluidics

Introduction

Single-cell RNA-sequencing (scRNA-seq) has become a cornerstone tool for studying cellular heterogeneity. However, most existing methods rely on oligo-dT priming, which exclusively captures polyadenylated RNA (polyA+). These methods are often limited to the length of reads marked with unique molecular identifiers (UMIs) for accurate deduplication, typically covering up to 600 bp from the 3′ end or, in some protocols, from the 5′ end. This leaves non-polyadenylated RNAs such as histone transcripts, non-coding RNAs, and enhancer RNAs undetected, thereby precluding accurate analysis of alternative splicing or RNA-based mutations. Several single cell total-RNA sequencing methods have been developed, such as Smart-seq-total [1], VASA-seq [2], and most recently TotalX [3]. These methods all rely on polyA-tailing of non-polyadenylated RNAs. While this allows the use of standard polyT-based single cell barcodes, it necessitates costly post-amplification ribosomal RNA (rRNA) removal. Among these, VASA-seq is the only method that captures full-length total RNA with UMI incorporation by fragmenting RNA prior to polyA-tailing. However, it requires a complex two-step droplet-merging process that is inaccessible to most microfluidics labs and incompatible with standard droplet-based platforms like 10X Genomics.

To address these limitations, we developed sc-rDSeq (single-cell ribosomal-depleted sequencing), a sensitive and affordable droplet-based protocol for full-length, strand-specific total RNA sequencing. The core of the method is a refined set of 220 “ribosomal-depleted sequences” (rDS) primers. These primers selectively exclude rRNA during reverse transcription, thus obviating the need for downstream rRNA depletion while capturing both polyadenylated and

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non-polyadenylated transcripts across the full length of each RNA molecule. The protocol builds on the widely used inDrops platform [4, 5] and requires only a single microfluidic step, making it accessible to labs familiar with droplet-based systems.

The sc-rDSeq workflow consists of three main stages: synthesis of barcoded rDS primers; single-cell co-encapsulation with barcoded beads for lysis and reverse transcription within droplets; and library construction, including second-strand synthesis, linear amplification via *in vitro* transcription, and strand-specific library preparation. The entire procedure from cell loading to a sequencing-ready library can be completed in approximately two days. Compared to polyA-targeted droplet methods (e.g., 10X Genomics, inDrops), sc-rDSeq has the potential to provide full-length coverage and detects non-polyadenylated RNAs, enabling analysis of splicing and non-coding transcriptomes (validation data are presented in the accompanying method article, 10.1093/nar/gkag312). Relative to plate-based full-length methods such as Smart-Seq2/3, sc-rDSeq can achieve higher throughput at a reported reagent cost of approximately $0.08 per cell (see 10.1093/nar/gkag312 for detailed benchmarking). Against total-RNA methods like VASA-seq or Smart-seq-total, sc-rDSeq avoids post-amplification rRNA depletion, employs a single-step microfluidic workflow, and is compatible with standard inDrops hardware, which may reduce experimental complexity and cost. A full quantitative comparison is provided in the accompanying method article (10.1093/nar/gkag312).

While highly versatile, the protocol's current rDS primer set is designed for human samples and will require redesign for other species. In addition, while sc-rDSeq uncovers population-level heterogeneity in alternative splicing, enhancer RNA (eRNAs), and single-nucleotide variations (SNVs), the detection of these events at ultra-high single-cell resolution remains sensitive to sequencing depth. We have successfully applied sc-rDSeq to profile drug-tolerant persister cells in lung cancer, revealing simultaneous changes in mRNA, histone RNA, miRNA, and eRNAs that were invisible to 3′ end-based methods. This protocol provides a comprehensive, step-by-step guide for sc-rDSeq, including reagent preparation, barcode synthesis, droplet generation, and library quality control.

Materials

Reagents

- Acrylamide, 40%, Merck, 01697
- Acrylamide/Bis-Acrylamide 19:1, 40%, Bio-Lab, 000135233100
- Ammonium persulfate (APS), Merck, A3678
- Tris-HCl 1M, pH 8.0, Bio-Lab, 208523
- Sodium chloride (NaCl), Sigma, S5150
- Potassium chloride (KCl), Sigma, P9541
- Ethylenediaminetetraacetic acid (EDTA), Biological Industries Israel, V4233
- Triton X-100, ENCO, 04807426
- Tween-20, ENCO, 04806576
- Isothermal amplification buffer, NEB, B0537
- Deoxynucleotide triphosphates (dNTP) Solution, NEB, N0446
- *Bst* 2.0® DNA Polymerase, NEB, M0537
- HFE-7500, RAN Biotechnologies, HFE7500-1500G
- 008-Fluorosurfactant, RAN Biotechnologies, 008-FluoroSurfactant-30G
- 1H, 1H, 2H, 2H-Perfluoro-1-octanol (PFO), Santa Cruz, sc-237819
- N, N, N′,N′-Tetramethyl ethylenediamine (TEMED), Bio-Lab, 20102399
- Sodium hydroxide (NaOH) solution, Merck, 1091371000
- Brij® L23 solution, Sigma, B4184
- Exonuclease I (ExoI), NEB, M0293
- OptiPrep, STEMCELL Technologies, 07820
- IGEPAL® CA-630, Sigma-Aldrich, I8896
- Murine RNase inhibitor, NEB, M0314
- AMPure XP beads, Beckman Coulter Life Sciences, A63881
- HinfI, NEB, R0155
- KAPA HiFi HotStart ReadyMix, Roche, KK2601
- High Sensitivity RNA ScreenTape, Agilent, 5067-5584
- High Sensitivity RNA ScreenTape Sample Buffer, Agilent, 5067-5580
- High Sensitivity D1000 ScreenTape, Agilent, 5067-5584
- High Sensitivity D1000 Sample Buffer, Agilent, 5067-5603

Table 1 Primer list.

Acrydite primer	/5ACryd/iSpPC/CGATGACGTAATACG ACTCACTATAGGGATACCACCATGGCT CTTTCCCTACACGACGCTCTTC
PE1[a]-BC1[a]-Spacer1[a]primer	AGCAATCACTC[8–10bp_Barcode1]AGAT CGGAAGAGCGTCGTGTAGGGAAAGAG
Spacer1[a]-BC2[a]-UMI[a]-Spacer2[a] primer	NNNTAGTAGACGACNNN[8bp_Barcode2] AGCAATCACTC
Spacer2[a]-UMI[a]-rDS[a] primer	[6bp_rDS]NNNTAGTAGACGAC
Spacer2[a]-UMI[a]-polyT5C8N[a] primer	NNNNNNNNCCCCCAAAAAAAAAAAANN NTAGTAGACGAC
PE2-N6-v2 primer	AGACGTGTGCTCTTCCGATCTNNNNNN
PE1 index primer	AATGATACGGCGACCACCGAGATCTACAC [8bp_Index1] ACACTCTTTCCCTACACGACGCTCTTCCGA TCT
PE2 index primer	CAAGCAGAAGACGGCATACGAGAT [8bp_Index2]GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATCT
T7-PE1-UMI-polyT	GATGACGTAATACGACTCACTATAGGGAT ACCACCATGGACACGACGCTCTTCCGATC TNNNNNNTTTTTTTTTTTTTTTTTV
T7-PE1-UMI-polyT5C8N	GATGACGTAATACGACTCACTATAGGGAT ACCACCATGGACACGACGCTCTTCCGATC TNNNNNNTTTTTTTTTTTGGGGGNNNN NNNN

[a] Represents reverse complement of the original sequence element. Full list of rDS sequences and barcodes can be found in Supplementary Material of Sun *et al*. (2026).

Primer sequences

Commercial kits

- StellarScript HT Reverse Transcriptase Kit, Watchmaker, WM-7K0070
- NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module, NEB, E6111
- HiScribe® T7 High Yield RNA Synthesis Kit, NEB, E2040
- RNA Fragmentation Reagents, Invitrogen, AM8740
- iQuant™ RNA HS Assay Kit, ABP Biosciences, N016
- iQuant™ dsDNA HS Assay Kit, ABP Biosciences, N011

Buffers and solutions

All primer sequences are listed in Table 1.

4x AB solution

Reagent	Final concentration	Amount
Acrylamide/Bis-Acrylamide 19:1, 40%	36%	3.6 ml
Acrylamide, 40%	26%	2.6 ml
nuclease-free water	n/a	3.8 ml
Total	n/a	10 ml

Note: Filter the solution through a 0.2 μm membrane, store at 4°C for up to 6 months.

10% APS

Reagent	Final concentration	Amount
APS	10%	0.1 g
nuclease-free water	n/a	1 ml
Total	n/a	1 ml

Note: Prepare fresh aliquot for each use.

Tris-buffered saline-EDTA-Triton (TBSET) buffer

Reagent	Final concentration	Amount
Tris-HCl [pH 8.0] 1 M	10 mM	10 ml
NaCl 1 M	137 mM	137 ml
KCl 2 M	2.7 mM	1.35 ml
EDTA 0.5 M	10 mM	20 ml
Triton X-100 10%	0.1%	10 ml
nuclease-free water	n/a	822 ml
Total	n/a	1000 ml

Note: Filter the solution through a 0.2 μm membrane, store at room temperature for up to 6 months.

Acrylamide beads mix

Reagent	Final concentration	Amount
4x AB solution	1x	250 μL
Acrydite primer 250 μM	100 μM	400 μL
10% APS	0.2%	20 μL
TBSET buffer	10%	100 μL
nuclease-free water	n/a	130 μL
Total	n/a	1000 μL

Note: Store at 4°C for up to 6 months.

2% HFE oil

Reagent	Final concentration	Amount
HFE-7500	n/a	2 g
008-Fluorosurfactant	2%	98 g
Total	n/a	100 g

Critical: Avoid direct contact with HFE-7500, it may cause respiratory, skin, and eye irritation.

Continuous phase oil

Reagent	Final concentration	Amount
TEMED	1.5%	60 μL
2% HFE oil	2%	4000 μL
Total	n/a	4060 μL

Critical: TEMED is highly toxic and flammable, handle inside a fume hood.
Note: Prepare fresh aliquot for each use.

$T_{10}E_{0.1}T_{0.1}$ buffer

Reagent	Final concentration	Amount
Tris-HCl [pH 8.0] 1 M	100 mM	500 μL
EDTA 0.5 M	0.1 mM	10 μL
Tween-20 10%	0.1%	500 μL
Nuclease-free water	n/a	49 ml
Total	n/a	50 ml

Note: Store at room temperature for up to 6 months.

Beads mix

Reagent	Final concentration	Amount
Hydrogel beads in T10E0.1T0.1 buffer	n/a	1.35 ml
Isothermal buffer 10X	2.5X	675 μL
dNTPs 25 mM	0.83 mM	90 μL
Nuclease-free water	n/a	585 μL
Total	n/a	2700 μL

Note: Prepare in dark or under UV-free light.

Bst mix

Reagent	Final concentration	Amount
Bst 2.0® DNA Polymerase 8 U/μL	0.2 U/μL	56.25 μL
Isothermal buffer 10X	1X	225 μL
dNTPs 25 mM	0.3 mM	27 μL
nuclease-free water	n/a	1.941 ml
Total	n/a	2250 μL

Stop solution

Reagent	Final concentration	Amount
Tris-HCl [pH 8.0] 1 M	10 mM	0.5 ml
KCl 2 M	100 mM	2.5 ml
EDTA 0.5 M	50 mM	5 ml
Tween-20 10%	0.1%	0.5 ml
nuclease-free water	n/a	41.5 ml
Total	n/a	50 ml

Note: Store at room temperature for up to 6 months.

NaOH buffer

Reagent	Final concentration	Amount
NaOH 1M	150 mM	6 ml
Brij 5%	0.5%	4 ml
Nuclease-free water	n/a	30 ml
Total	n/a	40 ml

Note: Store at room temperature for up to 6 months.

Neutralization buffer

Reagent	Final concentration	Amount
Tris-HCl [pH 8.0] 1 M	100 mM	5 ml
NaCl 5 M	100 mM	1 ml
EDTA 0.5 M	10 mM	1 ml
Tween-20 10%	0.1%	0.5 ml
nuclease-free water	n/a	42.5 ml
Total	n/a	50 ml

Note: Store in room temperature for up to 6 months.

rDS: polyT5C8N mix (4:1)

Reagent	Final concentration	Amount
Spacer2-UMI-rDS primer set 50 μM	50 μM	800 μL
Spacer2-UMI-polyT5C8N primer 50 μM	50 μM	200 μL
Total	n/a	1000 μL

Note: Store in small aliquots at −20°C for up to 12 months.

Primer attachment mix

Reagent	Final concentration	Amount
rDS: polyT5C8N mix (4:1)	37.5 μM	900 μL
Isothermal buffer 10X	1.25X	150 μL
dNTPs 25 mM	0.4 mM	20 μL
nuclease-free water	n/a	130 μL
Total	n/a	1200 μL

Primer extension mix

Reagent	Final concentration	Amount
Bst 2.0® DNA Polymerase 8 U/μL	0.2 U/μL	12.5 μL
Isothermal buffer 10X	1X	50 μL
dNTPs 25 mM	0.3 mM	6 μL
nuclease-free water	n/a	431.5 μL
Total	n/a	500 μL

ExoI treatment mix

Reagent	Final concentration	Amount
ExoI 20 U/μL	0.4 U/μL	11.5 μL
ExoI buffer 10X	1.5X	86 μL
nuclease-free water	n/a	470 μL
Total	n/a	567.5 μL

RT lysis mix

Reagent	Final concentration	Amount
StellarScript buffer 10X	2X	30 μL
IGEPAL CA-630 10%	0.6%	9 μL
dNTPs 25 mM	1 mM	6 μL

(continued)

(continued)

Reagent	Final concentration	Amount
Dithiothreitol (DTT) 0.1 M	6 mM	10 μL
Tris-HCl [pH 8.0] 1 M	20 mM	15 μL
Murine RNase inhibitor 40 U/μL	2.7 U/μL	10 μL
StellarScript HT RT enzyme 200 U/μL	20 U/μL	15 μL
Nuclease-free water	n/a	55 μL
Total	n/a	150 μL

Note: Prepare in 1.5 ml Eppendorf safe-lock tube.

Free primer digestion mix

Reagent	Final concentration	Amount
Post-RT product	n/a	70 μL
rSmartCut buffer 10X	2X	9 μL
ExoI 20 U/μL	0.6 U/μL	5 μL
HinfI 10 U/μL	0.4 U/μL	7 μL
Nuclease-free water	n/a	79 μL
Total	n/a	170 μL

Second strand cDNA synthesis mix

Reagent	Final concentration	Amount
Post-cleanup product	n/a	17 μL
NEB second strand synthesis buffer	n/a	2 μL
NEB second strand synthesis enzyme mix	n/a	1 μL
Total	n/a	20 μL

IVT mix

Reagent	Final concentration	Amount
Second-strand product	n/a	20 μL
T7 Reaction Buffer 10X	1X	8 μL
ATP 100 mM	10 mM	8 μL
CTP 100 mM	10 mM	8 μL
GTP 100 mM	10 mM	8 μL
UTP 100 mM	10 mM	8 μL
T7 RNA Polymerase Mix	n/a	8 μL
Nuclease-free water	n/a	12 μL
Total	n/a	80 μL

Random primer RT mix

Reagent	Final concentration	Amount
Post-fragmentation product	n/a	12 μL
PE2-N6-v2 primer 100 μM	10 μM	2 μL
StellarScript buffer 10X	1X	2 μL
dNTPs 10 mM	0.5 mM	1 μL
Dithiothreitol (DTT) 0.1 M	3 mM	1 μL
Murine RNase inhibitor 40 U/μL	2 U/μL	1 μL
StellarScript HT RT enzyme 200 U/μL	10 U/μL	1 μL
Total	n/a	20 μL

Equipment

- Microfluidic pressure control—LINEUP™ SERIES, Fluigent, LU-FEZ-0069; LU-FEZ-0345; LU-FEZ-1000; LU-FEZ-2000
- Hispec1 camera, FASTEC Imaging
- ECLIPSE Ti2-U inverted microscope, NIKON
- Liquid handling robot, Beckman Coulter Life Sciences, Biomek 4000
- High-Intensity UV Inspection Lamp, Capitol Scientific, 95-0127-01
- Magnetic stirring bar (2X2 mm), Spinbar, 371210008
- Micro gear motor, Firgelli Automation, FA-GM-6V-30
- Agilent 2200 TapeStation, Agilent, 2200
- Thermal cycler for 96-well plate, any type
- Thermal cycler for 384-well plate, any type
- Magnetic stand for AMPure XP beads, any type

Consumables

- Biomek® Modular Quarter reservoir, 19 ml/selection, Beckman Coulter, BK372788
- Biomek® Modular Quarter reservoir, 40 ml/selection, Beckman Coulter, BK372792
- pluriStrainer® Mini 70 μm (Cell Strainer), pluriSelect, 43-10070-40
- pluriStrainer® 40 μm (Cell Strainer), pluriSelect, 43-50040-51
- Minisart® NML 0.2 μm Cellulose Acetate Syringe Filter, Sartorius, 17823K
- Costar® Spin-X® Centrifuge Tube Filters, 0.45 μm Pore CA Membrane, Corning, 8163
- Eppendorf® Safe-Lock microcentrifuge tubes 1.5 ml, Merck, T9661
- Eppendorf® Safe-Lock microcentrifuge tubes 2 ml, Merck, T2795
- 96-well Polypropylene PCR Microplate, any type
- 384-well plate Polypropylene PCR Microplate, any type

Software

- OxyGEN, Fluigent, version 2.5.0

Methods

Step-by-step protocol

For complete details on the use and execution of this protocol, please refer to Sun *et al.* (2026) (DOI: 10.1093/nar/gkag312)[6].

The method includes three stages:

A. Production of sc-rDSeq barcoded hydrogel beads
B. Single-cell co-encapsulation with barcoded beads
C. sc-rDSeq library construction

Stage A can be performed in advance, and the barcoded beads can be stored for long-term use. The hydrogel beads are stable at 4°C protected from light for at least 6 months. Stages B and C should be performed consecutively with a possible stop point in the middle.

Preparation: microfluidic device fabrication and pump setup

Follow the protocol reported previously [7] to manufacture the poly (dimethyl siloxane) (PDMS) devices for barcode synthesis and single-cell co-encapsulation. CAD files for microfluidic devices can be found in Sun *et al.* (2026) (DOI: 10.1093/nar/gkag312) Supplementary Material.

Acrylamide beads production and single-cell co-encapsulation were performed using EZ pressure-derived pumps, controlled by the OxyGEN software at pressures ranging from 69 mbar to 2000 mbar. The flow was visualized under an optical microscope at 10 magnification and imaged at 1000–2000 frames per second using a Hispec1 camera.

A. Synthesis of sc-rDSeq barcoded hydrogel beads

Critical: Perform in darkness or under light that lacks UV wavelengths.

i. Acrylamide beads production (Day 1)
   1. Thaw an aliquot of Acrydite primer, prepare fresh **aqueous gel mix** and **continuous phase oil** in a 1.5 ml safe-lock tube and a 15 ml tube, respectively.
   2. Load inlets: Connect the inlet of **aqueous gel mix** to a 1000 mbar pump and the inlet of **continuous phase oil** tube to a 2000 mbar pump (Fig. 1).
   3. Prepare two collection tubes: 2 ml safe-lock Eppendorf tubes each containing 500 μL **continuous phase oil**.
   4. Turn on the pressure controllers, open the OxyGen control software. Change all the flow rate units to μL/h. Set scale factors for different inlets: 4.3 for oil.
   5. Start with 100 mbar for each inlet, then go to edit mode, set flow rate: 2000 μL/h **continuous phase oil**, 1000 μL/h **aqueous gel mix**, wait 10–15 s until the droplets formation is stable, move back to the pressure mode.
   6. Start collection. Collect for approximately 40 min per tube, or until the **aqueous gel mix** is exhausted.
   7. After collecting, close the collection tube and seal the opening with Parafilm, then cover with aluminum foil.
   8. Store horizontally, incubate in dark at room temperature overnight.
   9. Put collection tube vertically on a stand, wait 5 min for phase separation. Remove most of the oil from the bottom.
   10. Add 1 ml of 20% (v/v) PFO in HFE-7500 to break the emulsion.
   11. Vortex and centrifuge at 1500 rcf for 1 min. Remove the lower oil phase, leave the hydrogel in the bottom.
   12. Wash gels 3 times with 1 ml of **TBSET buffer**. Acrylamide beads can be stored in 1 ml **TBSET buffer** at 4°C in dark for at least 6 months. **Troubleshooting—Problem 1**

ii. Split-and-pool barcoding—Round 1 (Day 2)
   1. Wash Acrylamide beads twice in **$T_{10}E_{0.1}T_{0.1}$ buffer** by vortexing and centrifuging at 1500 rcf for 1 min, then remove the supernatant.
   2. Prepare the liquid handling working station: Place four 96-well plates containing BC1 primers (PE1*-BC1*-Spacer1* primer, one unique primer per well). Put a 384-well plate. Prepare **beads mix** in a 19 ml Reservoir.
      Critical: Do not mix between the cover of the primer plates, otherwise it introduces contamination to the primers in the well.
   3. Use robot to distribute 6 μL **beads mix** and 9 μL BC1 primer per well of 384-well plate.
   4. Take the 384-well plate to a thermal cycler, incubate at 85°C for 2 min, 60°C for 20 min. Put the plate back to the station.
   5. Prepare **Bst Mix** in a 19 ml Reservoir.
   6. Use robot to distribute 5 μL **Bst Mix** per well of 384-well plate.
   7. Take the 384-well plate to a thermal cycler, incubate at 60°C for 60 min. Put the plate back to the station.
   8. Prepare 20 ml **stop solution** in a 40 ml Reservoir.

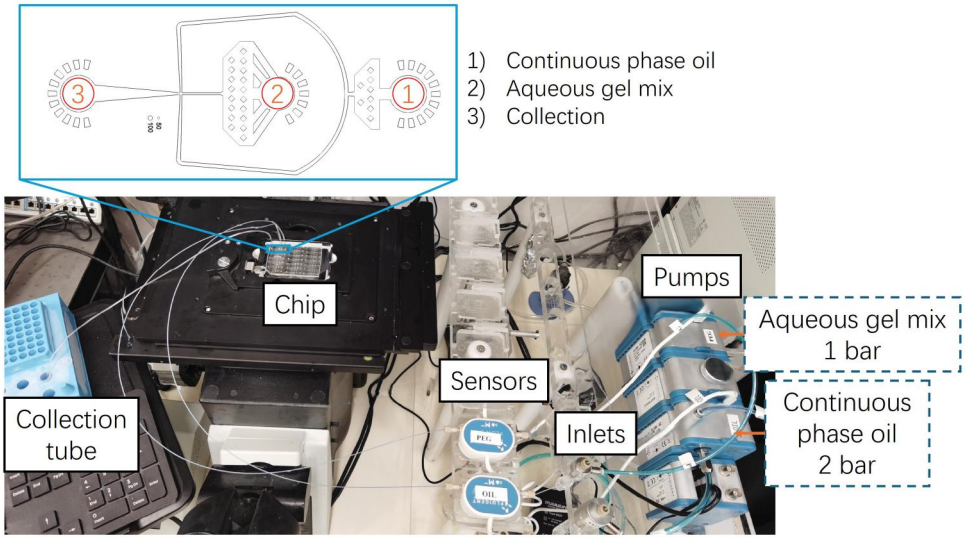

Figure 1 Acrylamide beads production microfluidics setup

9. Use robot to distribute 20 μL **stop solution** per well of 384-well plate. Incubate 10 min. Then collect all liquid in 384-well plate back to the 40 ml Reservoir.
10. Pour 40 ml Reservoir into a 50 ml tube. Rinse the reservoir wall with 1 ml **stop solution** and add to the tube.
11. Centrifuge at 1500 rcf for 1 min. Remove Supernatant.
12. Take 1 μL beads, resuspend in 9 μL nuclease-free water. Mark as "BC1." Keep in −20°C for later quality control.
13. Wash 3 times in 10 ml **NaOH buffer**. Incubate the last wash for 5 min.
14. Wash with 10 ml Neutralization buffer.
15. Wash 3 times in 10 ml **$T_{10}E_{0.1}T_{0.1}$ buffer**.
16. Resuspend in 10 ml **$T_{10}E_{0.1}T_{0.1}$ buffer**, wrap the tube with aluminum foil, store at 4°C.
17. Barcoded BC1 beads can be stored in 1 ml of **$T_{10}E_{0.1}T_{0.1}$ buffer** at 4°C in the dark for at least 6 months.

iii. Split-and-pool barcoding—Round 2 (Day 3)

1. Repeat as Round 1, replace BC1 with BC2 primer plates.
2. Before NaOH buffer wash, take 1 μL beads, resuspend in 9 μL nuclease-free water. Mark as "BC2." Keep in −20°C for later quality control.

iv. Split-and-pool barcoding—Round 3 (Day 4)

1. Take 300 μL BC2 beads, wash twice in T10E0.1T0.1 buffer by centrifuging at 1500 rcf for 1 min.
2. Prepare **rDS: polyT5C8N mix (4:1)**, then prepare 1200 μL Primer attachment mix.
3. Add **primer attachment mix** to BC2 beads and vortex thoroughly. Distribute the mixture evenly into 50 wells of a 96-well plate at 30 μL per well.
4. Take the 96-well plate to a thermal cycler, incubate at 85°C for 2 min, 60°C for 20min.
5. Prepare **primer extension mix**. Distribute the mixture evenly into 50 wells of a 96-well plate at 10 μL per well.
6. Take the 96-well plate to a thermal cycler, incubate at 60°C for 60 min.
7. Add 40 μL **stop solution** to each well. Incubate on ice for 30 min.
8. Collect all liquid into a reservoir, then into a 15 ml tube.
9. Centrifuge at 1500 rcf for 1 min. Remove supernatant.
10. Add 1 ml **$T_{10}E_{0.1}T_{0.1}$ buffer**, transfer beads into a 1.5 ml tube.
11. Take 1 μL beads, resuspend in 9 μL nuclease-free water. Mark as "BC3 before ExoI." Keep in −20°C for later quality control.

v. ExoI cleanup (Day 4)

1. Wash beads twice in **$T_{10}E_{0.1}T_{0.1}$ buffer** by centrifuging at 1500 rcf for 1 min.
2. Prepare **ExoI treatment Mix**, add to beads. Wrap tube with aluminum foil.
3. Rotate the tubes for 1 h at 37°C, protected from light.
4. Add 1 ml **Stop solution** and vortex.
5. Centrifuge at 1500 rcf for 1 min. Remove supernatant.
6. Take 1 μL beads, resuspend in 9 μL nuclease-free water. Mark as "BC3 after ExoI." Keep in −20°C for later quality control.
7. Wash 3 times in 1 ml **NaOH buffer**. Incubate the last wash for 5 min.
8. Wash with 1 ml Neutralization buffer.
9. Wash 3 times in 1 ml **$T_{10}E_{0.1}T_{0.1}$ buffer**.
10. Filter the barcode beads with pluriStrainer® Mini 70 μm.
11. Centrifuge at 1500 rcf for 1 min.
12. Take the supernatant to wash the pluriStrainer® Mini 70 μm. Repeat several times.
13. sc-rDSeq barcoded beads can be stored in 1 ml **$T_{10}E_{0.1}T_{0.1}$ buffer** at 4°C in dark for at least 6 months.
14. Resuspend in 1 ml **$T_{10}E_{0.1}T_{0.1}$ buffer**, sc-rDSeq barcoded beads can be stored in 1 ml **$T_{10}E_{0.1}T_{0.1}$ buffer** at 4°C in dark for at least 6 months.

vi. Barcode quality control (Day 4)

1. Thaw the beads saved from each round.
2. Release the primer by exposing the beads under UV for 10 min.
3. Centrifuge at 16 000 rcf for 5 min.
4. Run on 2% agarose gel.
5. Expected outcomes: BC1 88 nt, BC2 110 nt, BC3 120 nt, while "before ExoI" would have one clear band and a light smear at 50 nt, and "after ExoI" should only have a clear band. Expected gel photo is shown in Fig. 2. **Troubleshooting—Problem 2**

B. Single-cell encapsulation and reverse transcription (timing: 2–4 h)

i. Cell preparation

1. Filter OptiPrep through 0.2 μm filter.
2. Prepare single cell suspension in PBS according to the cell type specifications.
3. Pass cell suspension through a 40 μm cell strainer twice.
4. Make sure the viability is > 95%, otherwise wash the cell again.
5. Resuspend cells to 300k cells/ml in PBS with 10% OptiPrep in a 2 ml safe-lock Eppendorf.
6. Take a magnetic stirring bar, wash 3 times in PBS, put it in the tube with the cells.
7. Hold the tube on ice until use.

ii. Microfluidics preparation

1. Prepare **RT lysis mix**.
2. Prepare carrier oil: HFE-7500 with 2% 008-Fluorosurfactant. Take 3 ml carrier oil in a 15 ml tube.
3. Prepare collection plate: take a 96-well plate, fill each well for collection with 50 μL carrier oil. Cover the plate with aluminum foil until collection starts.

   Critical: Perform in darkness or under light that lacks UV wavelengths.

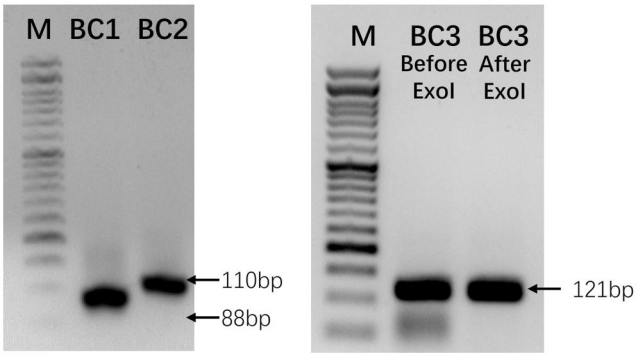

Figure 2 sc-rDSeq barcoded beads quality control expected outcomes

4. Take 300 μL sc-rDSeq barcoded beads, centrifuge at 1500 rcf for 2 min, remove buffer.
5. Load inlets: connect carrier oil, cell, **RT lysis mix** and barcode beads to 69 mbar, 345 mbar, 345 mbar and 1000 mbar pumps, respectively (Fig. 3).
6. Turn on the rotating motor for the magnetic stirring bar in the cell suspension to prevent sedimentation and clumping.

iii. Single-cell encapsulation and reverse transcription

1. Turn on the pressure controllers, open the OxyGen control software. Change all the flow rate units to μL/h. Set scale factors for different inlets: 4.3 for oil.
2. Start with oil—69 mbar, lysis—20 mbar, cells—30 mbar, barcodes—50 mbar, then go to edit mode, set flow rate mode: oil—360 μL/h, cells—250 μL/h, lysis—250 μL/h, wait 10–15 s until droplet formation is stable. Then start pushing barcodes to achieve a state where every oil droplet contains a barcode. Make sure drops size to be around 2 nL. **Troubleshooting—Problem 3**
3. Take a short video (3–10 s) to calculate how many cells are encapsulated in a minute. **Troubleshooting—Problem 4**
4. Collect about 1500 cells per well, which takes about 15–20 mins. Repeat for 2–4 collections per sample.
   Troubleshooting—Problem 5
5. After collection, transfer the collection plate to UV for 10 min.
6. Recycle the unused barcoded beads with **$T_{10}E_{0.1}T_{0.1}$ buffer**.
7. Transfer the collection plate to a thermal cycler, perform ramping-cycling RT: 20 cycles of (8°C for 12 s, 15°C for 45 s, 20°C for 45 s, 30°C for 30 s, 50°C for 15 s).
8. Take the collection plate to room temperature, remove HFE-7500 (bottom phase), then transfer the contents of each well into separate 1.5 ml tubes.
9. Add 20% (vol/vol) PFO in HFE-7500 to each collection, lightly flick the tube, wait to see the emulsion break into two separate clear phases.

   Stopping point: Store post-RT libraries at −80°C. They are stable for at least 3 months.

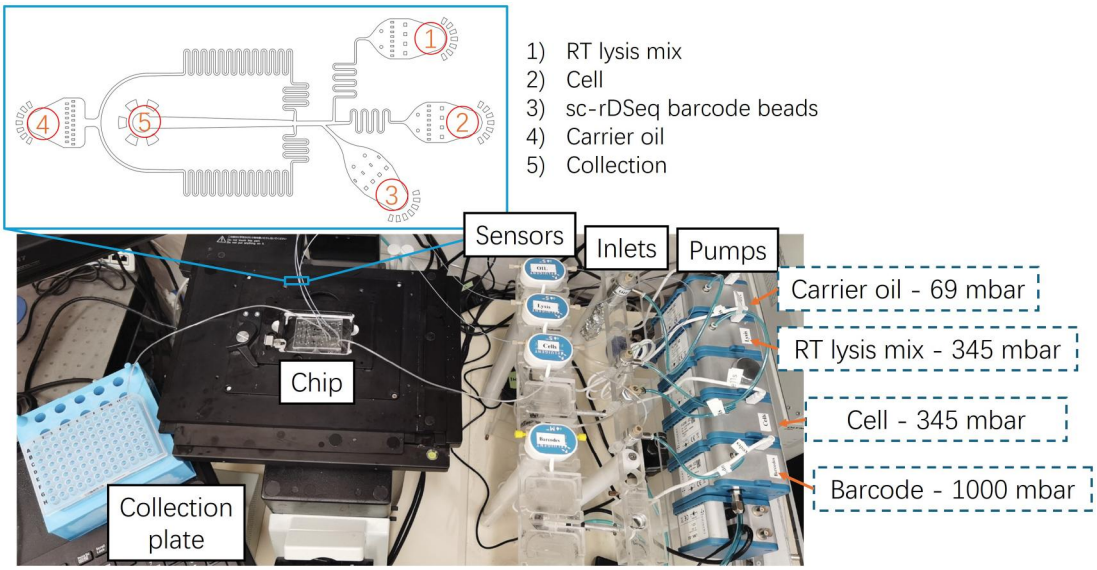

Figure 3 sc-rDSeq microfluidics setup

C. sc-rDSeq library construction (timing: 2 days)

i. Emulsion breakage and free primer digestion
   1. Thaw post-RT libraries on ice. Centrifuge the tubes at 4°C for 5 min at 19 000 rcf.
   2. Transfer the post-RT material (top phase) to Spin-X column and collect the flow-through fraction into a 1.5 ml DNA LoBind tube by centrifuging at 16 000 rcf for 1 min at 4°C. Keep the original tube with HFE-7500 oil for later use.
   3. After centrifugation, leave the filter column inserted into the collection tube.
   4. Measure sample volume. For each 70 μL of post-RT material, prepare 100 μL of **free primer digestion mix**.
   5. Add **free primer digestion mix** on top of the HFE-7500 oil in the original 1.5 ml tube and mix gently by flicking the tube. Quickly centrifuge to collect all liquid on inner wall of the tube.
   6. Transfer the **free primer digestion mix** with the remaining emulsion to the Spin-X column containing the bulk of the sample and centrifuge the filter column at 16 000 rcf for 5 min at 4°C.
   7. Incubate the flow-through fraction at 37°C for 30 min in a heating block.
   8. Purify with 1.2X AMPure beads, elute in 17 μL Tris-HCl 10 mM, transfer to a new 96-well plate.

ii. Second-strand synthesis (SSS)
   1. Prepare Second strand cDNA synthesis mix on ice.
   2. Add 3 μL of **second strand cDNA synthesis mix** to 17 μL of post-cleanup product.
   3. Incubate at 16°C for 2.5 h, then at 65°C for 20 min.

iii. In vitro transcription (IVT)
   1. Prepare **IVT mix** on ice.
   2. Add 60 μL of **IVT mix** to 20 μL of post-SSS product.
   3. Incubate at 37°C for 13–15 h, then hold at 4°C for up to 2 h.
   4. Purify with 1.3X AMPure beads, elute amplified RNA (aRNA) in 20 μL Tris-HCl 10 mM, transfer to a new 1.5 ml tube.
   5. Take 1 μL aRNA to measure concentration with Qubit, expect > 10 ng/μL. **Troubleshooting—Problem 6**
   6. Run 1 μL on Agilent 2200 TapeStation High Sensitivity D1000, expect a peak at 100 nt as uncleaned barcoded primer, and a smear of 150–1000 nt. Example can be seen as follow in Fig. 4.

   Stopping point: Store aRNA at −80°C. They are stable for at least 6 months.

iv. RNA fragmentation
   1. Thaw aRNA on ice. Transfer to a new 96 well-plate.
   2. Mix 9 μL aRNA with 1 μL RNA fragmentation reagent.
   3. Incubate at 70°C for 1 min, then immediately place on ice.
   4. Add 34 μL fragmentation stop mix: 1.2 μL stop solution from RNA fragmentation reagent kit, 9.8 μL Tris-HCl 10 mM and 26.4 μL AMPure beads.
   5. Incubate 5 min on ice.
   6. Put plate on magnet, continue with washing, elute in 12 μL Tris-HCl 10 mM.

v. Random primer RT
   1. Prepare Random primer RT mix on ice.
   2. Add 8 μL of **random primer RT mix** to 12 μL of post-fragmentation product.
   3. Incubate at 25°C for 10 min, 50°C for 60 min, 80°C for 10 min.
   4. Add 20 μL nuclease-free water, mix well.
   5. Transfer 20 μL of pre-PCR product into a new 1.5 ml tube for back-up.

   Stopping point: Store pre-PCR product at −80°C. They are stable for at least 6 months.
   6. Purify with 1.2X AMPure beads, elute cDNA in 11.5 μL Tris-HCl 10 mM.

vi. PCR amplification
   1. Choose index primers according to sequencing needs.
   2. Add 12.5 μL KAPA HiFi HotStart ReadyMix, 0.5 μL of 25 μM PE1 index primer and 0.5 μL of 25 μM PE2 index primer.
   3. Incubate for cycles: 98°C for 2 min; 2 cycles of (98°C for 20 s, 55°C for 30 s, 72°C for 40 s); 8–12 cycles of (98°C for 20 s, 65°C for 30 s, 72°C for 40 s); 72°C for 10 min.
   4. Add 25 μL nuclease-free water, mix well.
   5. Purify with 0.6X AMPure beads, elute DNA in 20 μL Tris-HCl 10 mM.
   6. Take 1 μL DNA to measure concentration with Qubit, expect 1–10 ng/μL. **Troubleshooting—Problem 7**
   7. Run 1 μL DNA on Agilent 2200 TapeStation High Sensitivity RNA, expect a smear of 300–1000 nt. Example can be seen as follow in Fig. 5. **Troubleshooting—Problem 8**
   8. The library is ready for sequencing. The recommended sequencing parameters are: Read 1 70 bp, Read 2 > 30 bp, Index 1 8 bp, Index 2 8 bp.

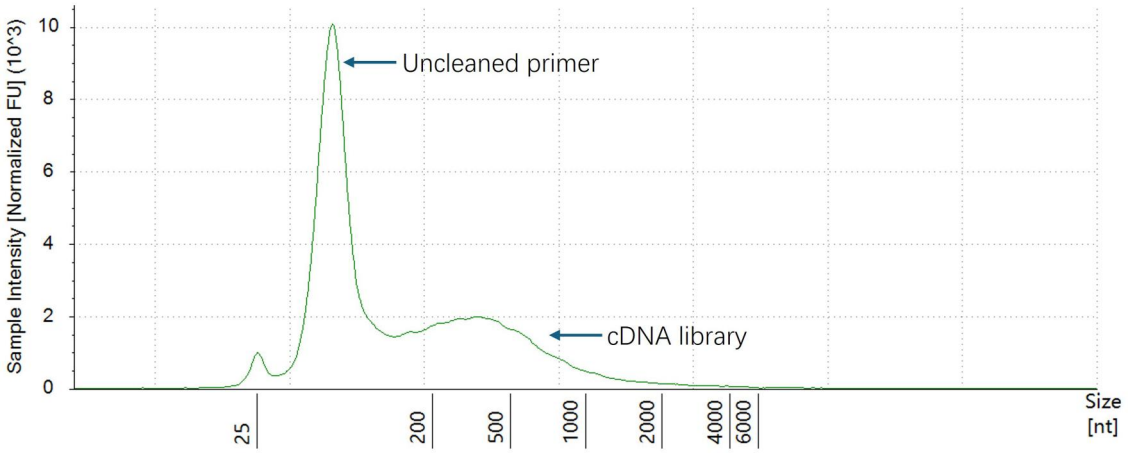

Figure 4 IVT TapeStation example

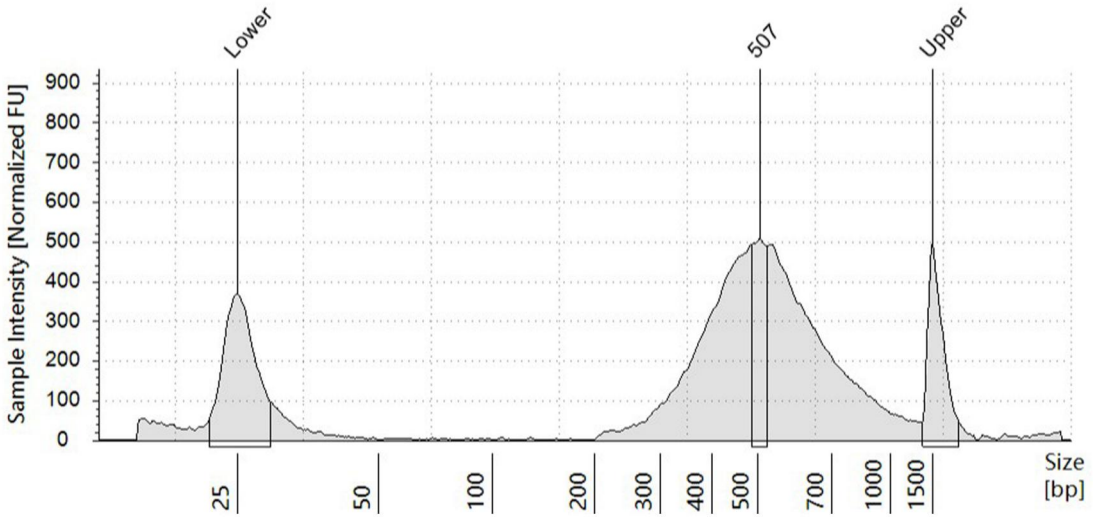

Figure 5 PCR TapeStation example

Data analysis

This protocol describes the raw data processing steps aimed at obtaining single-cell total-RNA expression matrices. The code for raw data processing is available online at https://doi.org/10.6084/m9.figshare.29163206, and a detailed step-by-step instruction is provided in Supplementary Material. Here we only describe the brief workflow.

FASTQ raw files generated by the sequencing platform are processed to generate a single-cell gene expression matrix. The required code can be found at the above DOI and includes the following steps:

1. Pre-processing data QC: Check data quality, output per barcode reads distribution histogram to decide UMI threshold.
2. Demultiplexing: Process single cell barcode and UMI information (R1), output expression fastqs per single cell (R2).
3. Adaptor trimming and rRNA filtering: Trim Illumina adaptors and sequence handles, filter out human rRNA reads based on NCBI rRNA sequences.
4. Align reads in the following order: small RNA, long RNA exons, long RNA introns, and pseudogenes. Only reads that fail to align in a given round proceed to the next round. After alignments, the counting matrix of each step is generated.

From here, users can take the single-cell expression matrix for downstream analysis using commonly used packages such as Seurat or Scanpy. Additional downstream analyses can be performed according to the scope of the research.

Limitations

The current rDS primer set was designed specifically for the human transcriptome; applying this protocol to other species will require redesigning the rDS primer pool to match the ribosomal RNA sequences of that species. Moreover, the protocol has been validated primarily in cultured cancer cell lines; its performance in primary tissues, clinical samples, or cells with very low RNA content has not been extensively characterized and may require optimization of lysis or reverse transcription conditions. Users are advised to perform regular quality checks on beads, microfluidics chips, and UV equipment, and to include spike-in controls whenever adapting the protocol to new sample types.

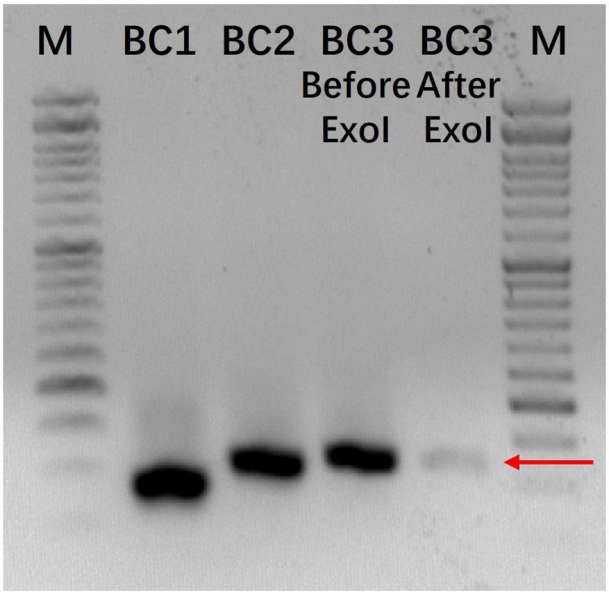

Figure 6 Example of inefficient primer synthesis

Troubleshooting

Problem 1

During acrylamide beads production, no hydrogel formed from first centrifuge after breaking emulsion.

Potential solution: Replace 4x AB with fresh solution. Prepare APS on the day of experiment.

Problem 2

Too much loss of primers after ExoI treatment during sc-rDSeq barcode synthesis. The quality control would show up as the band of "BC3 after ExoI" to be much fainter than "BC3 before ExoI" (Fig. 6). It

means most of primers were single strands before NaOH treatment and meant inefficient synthesis of primers during the third round of barcoding.

Potential solution: The first step of distributing **primer attachment mix** and barcode beads mixture into 96-well plate improves the efficiency of the primer synthesis. To improve efficiency, ensure thorough mixing when distributing the Primer attachment mix and barcode beads into the 96-well plate. Also ensure that the entire process is performed in darkness or under light that lacks UV wavelengths.

Problem 3

During single-cell encapsulation, the barcoded beads come in groups instead of one by one.

Potential solution: Wash the beads twice with $\mathbf{T_{10}E_{0.1}T_{0.1}}$ **buffer**, and pass the beads through 70 μm strainer again.

Problem 4

Don't see cells coming from the inlets, or less than one cell is encapsulated per second.

Potential solution: If cells are small (ex. Embryonic stem cells, immune cells, etc.), consider change the focus and zoom in the microscope. If still don't see enough cells, try to flip lightly the needle for cell inlets, there might be a blockage.

It is not recommended to use a too diluted cell suspension for sc-rDSeq. Due to the high sensitivity of the rDS primers to all RNA, including ambient RNA which introduce noise, it is suggested to minimize the number of empty drops as much as possible.

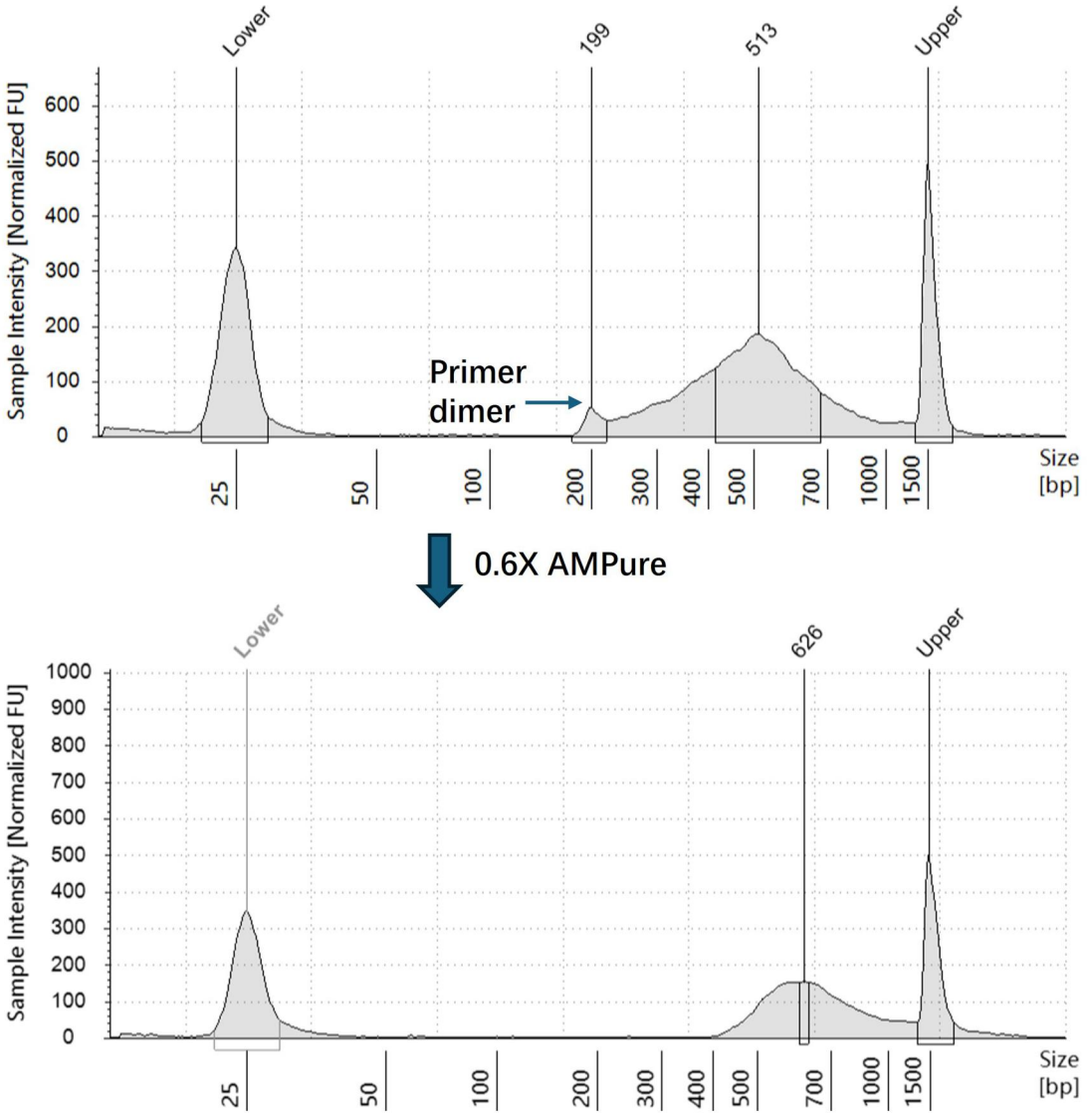

Figure 7 Example of primer dimer in library (top), which could be removed with 0.6X AMPure (bottom)

Problem 5

Droplet coalescence or premature breakage during handling.

Potential solution: Make sure all tubing, channels and needles are clean. Use a flashlight to check the collection tubing, if a discontinue emulsion is observed, change to a new channel, with new collection tubing and needle.

Problem 6

After IVT, the RNA concentration is too low (< 10 ng/μL) or too high (> 100 ng/μL).

Potential solution: If the RNA concentration is too low, it means either the cells were not in a good state, or the previous steps were not performed correctly. Try troubleshooting previous steps, including barcode beads production.

If the RNA concentration is too high, usually it means too many unremoved primers in the library, which can be confirmed by TapeStation results. Try reducing the volume of collection and consider performing extra round of Spin-X column filtering.

Problem 7

After PCR, the DNA concentration is too low (< 1 ng/μL).

Potential solution: Go back to the "pre-PCR" backup, increase the number of cycles of PCR to maximum 2 + 12 cycles.

Problem 8

TapeStation of PCR product shows a large amount of primer dimers. As shown in Fig. 7.

Potential solution: Perform another round of 0.6X AMPure for strict size selection. If the PCR product is low in concentration (<5 ng/μL), consider eluting in smaller volume (e.g., 10 μL), or perform another round of PCR with the corresponding "post-PCR" back-up.

Problem 9

When applying to primary tissue or new cell system, if all troubleshooting steps are performed, the library quality is still not okay.

Potential solution: Optimize lysis and reverse transcription conditions for the target sample type; Perform regular quality checks on beads, microfluidic chips, and UV equipment; Include spike-in controls when testing new sample types.

Quick reference: critical steps and common failure points

While the Troubleshooting section above addresses specific symptoms, the steps listed below are the most common points of failure encountered by new users. We have flagged them in the main protocol with the notation **Critical**. Reviewing this table before starting the experiment will help you allocate extra attention and preparation time to the highest-risk steps.

Core principles for maximizing success on the first attempt

1. **Test-run on a reference sample.** Before applying sc-rDSeq to precious or rare primary samples, we strongly recommend performing a complete test run on a well-characterized cell line (e.g., HEK293T or HeLa) using the same reagent lots. This serves as a positive control for reagent integrity, bead quality, and microfluidic performance.
2. **Avoid interruptions at CRUCIAL steps.** Once droplets are generated, the protocol proceeds through several interdependent enzymatic steps. Plan your workflow so that these steps can be completed in a single session, without overnight pauses, whenever possible.
3. **Use spike-in controls when adapting to new sample types.** Spike-in RNA (e.g., ERCC) added at the lysis step can help normalize for technical variation and diagnose sample-specific failures independently of the biological RNA content.

Step	Most common failure mode	Key recommendation
sc-rDSeq barcode synthesis	Excessive primer loss after ExoI treatment, indicating inefficient third-round barcoding	Ensure thorough mixing of the Primer Attachment Mix with barcode beads during plate distribution. Perform the entire synthesis in darkness or under UV-free light to prevent photodamage.
Microfluidic chip and bead quality check	Clogging, inconsistent droplet size, or bead aggregation	Always inspect chips under a microscope before use. Pre-wash beads and filter all solutions immediately prior to loading.
Cell loading and droplet generation	Incorrect cell concentration leads to high doublet rate or many empty droplets	Count cells carefully and prepare a fresh dilution immediately before encapsulation. Acquire multiple videos during droplet collection and average cell counts across fields for more reliable estimation.
Droplet collection and integrity	Droplet coalescence or premature breakage during handling	Make sure all tubing, channels and needles are clean. Use a flashlight to check the collection tubing, if a discontinue emulsion is observed, change to a new channel, with new collection tubing and needle.
Primer removal	Incomplete removal causes short-library contamination	Ensure that the cell encapsulation rate is adequate (≥ 1 cell/sec) to minimize empty droplets carrying excess free primers; consider additional Spin-X column filtering if a strong primer peak persists.
IVT and PCR yield	Low yield or over-amplification bias	Always use freshly prepared reagents and calibrate the number of PCR cycles using a small-scale pilot run when processing a new sample type.

Author contributions

Conceptualization and methodology, Xue Sun and Oren Ram; Investigation and visualization, Xue Sun; Writing—original draft, Xue

Sun; Writing—review & editing, Xue Sun and Oren Ram; Resources, Funding acquisition and Supervision, Oren Ram.

Supplementary material

Supplementary material is available at *Biology Methods and Protocols* online.

Conflicts of interest

None declared.

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Data availability

The accession number for the data reported in this paper is GEO: GSE282074. The code used to analyze the data are available at https://doi.org/10.6084/m9.figshare.29163206.

AI disclosure

During the preparation of this work, the authors used Gemini and DeepSeek. These AI tools were employed to improve spelling, grammar, and readability. Edits were made by submitting each sentence separately. Subsequently, the authors reviewed and edited each sentence as needed and take full responsibility for the content of the published article.

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, O.R. (oren.ram@mail.huji.ac.il).

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