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A rapid, sensitive, scalable method for Precision Run-On sequencing (qPRO-seq)

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We use this protocol and it's working

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Materials

MATERIALS

- ThermoPol Reaction Buffer Pack - 6.0 ml **New England Biolabs Catalog #**B9004S
- T4 RNA Ligase 1 (ssRNA Ligase) - 5,000 units **New England Biolabs Catalog #**M0204L
- RNA 5′ Pyrophosphohydrolase (RppH) - 200 units **New England Biolabs Catalog #**M0356S
- Q5 High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #**M0491S
- SYBR Gold Nucleic Acid Gel Stain **Catalog #** S-11494
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- Magnesium Chloride **Fisher Scientific Catalog #**AC223210010
- TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026
- EGTA **Merck MilliporeSigma (Sigma-Aldrich)**
- Superase-In RNase Inhibitor **Thermofisher Catalog #**AM2694
- sarkosyl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L5777
- Sucrose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S7903
- Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5758
- Chloroform **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**319988
- Sodium hydroxide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S8045
- Potassium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9541
- Glycerol **Thermo Fisher Scientific Catalog #**17904
- Dynabeads MyOne Streptavidin C1 **Invitrogen - Thermo Fisher Catalog #**65001
- Dithiothreitol (DTT) **Thermo Fisher Scientific Catalog #**707265ML
- Tris Base **Fisher Scientific Catalog #**BP152
- T4 Polynucleotide Kinase **New England Biolabs Catalog #**M0201S
- Triton X-100 **Fisher Scientific Catalog #**BP151-100
- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9416
- Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S3014
- Ethanol **Merck Millipore (EMD Millipore) Catalog #**100983
- TRIzol™ LS Reagent **Thermo Fisher Catalog #**10296028
- EDTA **Thermo Fisher Catalog #**17892
- ATP **Thermo Fisher Catalog #**18330019
- GTP **Thermo Fisher Catalog #**18332015

- Pierce Protease Inhibitor Tablets **Thermo Fisher** **Catalog #**A32963
- GlycoBlue™ Coprecipitant (15 mg/mL) **Thermo Fisher** **Catalog #**AM9515
- DEPC-Treated Water **Thermo Fisher** **Catalog #**AM9920
- Maxima H Minus Reverse Transcriptase (200 U/µL) **Thermo Fisher** **Catalog #**EP0753
- IGEPAL® CA-630 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I8896
- SsoAdvanced Universal SYBR® Green Supermix **Bio-Rad Laboratories** **Catalog #**172-5270
- Trypan Blue **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T8154
- Biotin-11-CTP **Perkin Elmer** **Catalog #**NEL542001EA
- Biotin-11-UTP **Perkin Elmer** **Catalog #**NEL543001EA
- Biotin-11-GTP **Perkin Elmer** **Catalog #**NEL545001EA
- Biotin-11-ATP **Perkin Elmer** **Catalog #**NEL544001EA
- Total RNA Purification Kit **Norgen Biotek Corp.** **Catalog #**37500
- dNTP mix 12.5 mM each **Roche** **Catalog #**03622614001
- Micro Bio-Spin RNase free P-30 Gel Columns **Bio-Rad Laboratories** **Catalog #**7326250
- Costar Spin-X 0.22 um Centrifuge Tube Filters **Corning** **Catalog #**1860
- REV3 Adapter /5Phos/rUrNrNrNrNrNNGATCGTCGGACTGTAGAACTCTGAAC/3InvdT/ RNase-free HPLC **IDT**
- REV5 adapter /5InvddT/CCTTGGCACCCGAGAATTCCANrNrNrNrNrNrC RNase-free HPLC **IDT**
- Primer RP1 AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA PAGE-purified **IDT**
- Primer RPI-1 CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-2 CAAGCAGAAGACGGCATACGAGATAGATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-3 CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-4 CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-5 CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-6 CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**
- Primer RPI-7 CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified

Primer RPI-8 CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**

Primer RPI-9 CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**

Primer RPI-10 CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**

Primer RPI-11 CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**

Primer RPI-12 CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA PAGE-purified **IDT**

Cell Permeabilization

1h 30m

1 Prepare permeabilization buffer, wash buffer, and freeze buffer and place On ice .

Safety information

CAUTION: DEPC is toxic and harmful!

Safety information

CRITICAL: Care should be taken to avoid nuclease contamination. Change gloves routinely and prepare/use nuclease-free reagents.

Safety information

CRITICAL: ALL steps should be carried out on ice or in a cold room

Note

All salt solutions should be prepared in ddH_2O. Then add 0.1% (v/v) DEPC, stir overnight, and autoclave. Tris buffers instead need to be carefully prepared with DEPC-treated ddH_2O.

Note

All other solutions (detergents, DTT, sucrose, EDTA/EGTA, and Tris buffers) should be prepared in DEPC ddH_2O in RNase free containers and filter sterilized. Glassware can be made RNase by filling with water, adding 0.1% (v/v) DEPC, incubating with agitation overnight, and autoclaving. Alternatively, glassware can be baked at 300 °C for 4 hours.

Note

The permeabilization buffer, cell wash buffer, freeze buffer, and bead washing/binding buffers can be made and filter-sterilized in advance without the DTT, SUPERase-In™ RNase Inhibitor, and Pierce™ protease inhibitor tablets. DTT, SUPERase-In™ RNase Inhibitor, and protease inhibitor tablets can be added when buffers are needed. Store buffers at 4°C. Use DEPC treated glassware or RNase free plasticware.

1.1 **Permeabilization Buffer:**

[M] 10 millimolar (mM) Tris-Cl, pH 8.0

[M] 10 millimolar (mM) KCl

[M] 250 millimolar (mM) Sucrose

[M] 5 millimolar (mM) $MgCl_2$

[M] 1 millimolar (mM) EGTA

[M] 0.1 % (v/v) Igepal

[M] 0.5 millimolar (mM) DTT

[M] 0.05 % (v/v) Tween-20

[M] 10 % (v/v) Glycerol

in DEPC-treated ddH_2O.

Add 1 Pierce protease inhibitor tablet and 10 µL SUPERase-In RNase inhibitor per 50 mL.

1.2 **Cell Wash buffer:**

[M] 10 millimolar (mM) Tris-Cl, pH 8.0

[M] 10 millimolar (mM) KCl

[M] 250 millimolar (mM) sucrose

[M] 5 millimolar (mM) $MgCl_2$

[M] 1 millimolar (mM) EGTA

[M] 0.5 millimolar (mM) DTT

[M] 10 % (v/v) Glycerol

in DEPC-treated ddH_2O.

Add 1 Pierce protease Inhibitor tablet and 10 µL SUPERase-In RNase inhibitor per 50 mL.

1.3 **Freeze buffer:**

[M] 50 millimolar (mM) Tris-Cl, pH 8.0

[M] 40 % (v/v) glycerol

[M] 5 millimolar (mM) $MgCl_2$

[M] 1.1 millimolar (mM) EDTA

[M] 0.5 millimolar (mM) DTT

in DEPC-treated ddH_2O.

Add 10 µL SUPERase-In RNase inhibitor per 50 mL .

2 Proceed using one of the following options:

2.1 **Option 2.1.: Adherent cells (volumes are for 10 cm plates):**

2.1.1. Wash cells with 10 mL ice cold PBS.

2.1.2. Repeat the PBS wash step for a total of two washes.

2.1.3. Add 5 mL ice cold permeabilization buffer, scrape cells, and transfer to a conical tube.

2.1.4. Rinse plate with 5 mL permeabilization buffer and pool cells in conical tube (V_f = 10 mL).

2.2 **Option 2.2: Suspension cells:**

2.2.1. Transfer cells into conical tubes and spin down at 700–1000 x g for 00:04:00 at 4 °C .

2.2.2. Wash with 10 mL ice cold PBS.

2.2.3. Repeat the PBS wash for a total of two washes.

2.2.4. Resuspend in 10 mL cold permeabilization buffer.

Note

Use a centrifuge with a swinging bucket rotor for all centrifuge steps during cell permeabilization. Using a fixed angle rotor will shear cells, releasing a smear of white chromatin.

Note

Centrifuge speed is cell size dependent. We typically centrifuge HeLa at 800 x g and Drosophila at 1,000 x g.

Note

When resuspending cells during permeabilization after centrifugation steps, first gently resuspend the cell pellet with 1 mL solution with a wide-bore P1000 tip. Then add the remaining volume (usually 9 mL) and mix by gentle inversion.

3 Incubate on ice for 00:05:00 .

4 Check for permeabilization with Trypan blue. Greater than 98% permeabilization is ideal.

Note

If your cell type is not permeabilized under these conditions, add Triton X-100 to [M] 0.1 % (v/v) – [M] 0.2 % (v/v) .

5 Spin down at 700–1000 x g for 00:04:00 at 4 °C .

Note

Centrifuge speed is cell size dependent. We typically centrifuge HeLa at 800 x g and Drosophila at 1,000 x g.

6 Wash with 10 mL ice cold cell wash buffer.

Note

When resuspending cells during permeabilization after centrifugation steps, first gently resuspend the cell pellet with 1 mL solution with a wide-bore P1000 tip. Then add the remaining volume (usually 9 mL) and mix by gentle inversion.

7 Repeat the cell wash buffer wash for a total of two washes.

8 Decant wash buffer, and then carefully pipette off remaining buffer and discard without disturbing the cell pellet.

9 Using wide-bore tips, resuspend in 250 µL cold freeze buffer and transfer to a 1.5 mL tube.

10 Rinse the conical tube with an additional 250 µL freeze buffer and pool (V_f = 500 µL).

11 Count cells and add permeabilized spike-in cells if desired.

Note

When processing multiple samples, if counting will cause the cells to sit on ice for greater than 10 min, reserve 10 µL for counting, aliquot cells in 100 µL aliquots, and snap freeze. Count the cells and then adjust the concentration with freeze buffer after thawing and prior to the run-on.

Note

In order to robustly normalize between conditions where a dramatic change in global transcription levels are expected, we add a fixed number of cells of a different species to a fixed number of experimental cells at the permeabilization step. Reads can be mapped to a combined genome, and the number of spike-in mapped reads can then be used as a scaling factor. These cells should be permeabilized prior to the experiment, aliquoted, and added to 1-2% by cell number after permeabilization and counting, either just prior to freezing or just prior to the run-on reaction. We frequently use Drosophila S2 cells to normalize human cell experiments and vice versa.

12 Spin down at 1000 x g for 00:05:00 at 4 °C.

Note

Microfuge tubes can be spun in a fixed angle rotor, but we continue to use a swinging bucket rotor so that cells collect at bottom of tube (this tends to decrease cell loss).

13 Resuspend the desired number of cells for each run-on reaction in 52 µL freeze buffer.

Note

We have had success performing this protocol with as few as 50k primary human cells. In general, we find that the quality of libraries will increase until ~1 x 10^6 cells per run-on but using more cells than this offers little benefit. This will also depend on how transcriptionally active a given cell type is and genome size.

14 Continue to the run-on or snap freeze 52 µL aliquots in LN_2 and store at -80 °C .

Note

Permeabilized cells are stable indefinitely at -80°C (Chu et al., 2018).

Preparation for the Run-On

30m

15 Pre-chill a microcentrifuge to 4 °C .

16 Set a heat block with water in the wells to 37 °C and another to 65 °C and allow temperature to equilibrate.

Note

A thermomixer set to 37 °C can also be used for incubating the run-on reactions.

17 Prepare bead preparation buffer, high salt wash buffer, low salt wash buffer, and binding buffer, and store On ice

Note

The permeabilization buffer, cell wash buffer, freeze buffer, and bead washing/binding buffers can be made and filter-sterilized in advance without the DTT, SUPERase-In™ RNase Inhibitor, and Pierce™ protease inhibitor tablets. DTT, SUPERase-In™ RNase Inhibitor, and protease inhibitor tablets can be added when buffers are needed. Store buffers at 4°C. Use DEPC treated glassware or RNase free plasticware.

17.1 **Bead Preparation Buffer:**

[M] 0.1 Mass Percent NaOH

[M] 50 millimolar (mM) NaCl

in DEPC-treated ddH_2O.

17.2 **Bead Binding Buffer:**

[M] 10 millimolar (mM) Tris-HCl, pH 7.4

[M] 300 millimolar (mM) NaCl

[M] 0.1 % (v/v) Triton X-100

[M] 1 millimolar (mM) EDTA

in DEPC-treated ddH_2O.

Add 2 µL SUPERase-In RNase Inhibitor per 10 mL.

17.3 **High Salt Wash buffer:**

[M] 50 millimolar (mM) Tris-HCl, pH 7.4

[M] 2 Mass Percent NaCl

[M] 0.5 % (v/v) Triton X-100

[M] 1 millimolar (mM) EDTA

in DEPC-treat H_2O.

Add 2 µL SUPERase-In RNase Inhibitor per 10 mL.

17.4 **Low Salt Wash Buffer:**

[M] 5 millimolar (mM) Tris-HCl, pH 7.4

[M] 0.1 % (v/v) Triton X-100

[M] 1 millimolar (mM) EDTA

in DEPC-treated ddH_2O.

Add 2 µL SUPERase-In RNase Inhibitor per 10 mL.

18 For each run-on reaction, wash 10 µL Dynabeads™ MyOne™ Streptavidin C1 Beads once in 1 mL bead preparation buffer using a magnet stand. Beads can be washed in bulk.

Safety information

CRITICAL:Be sure to properly resuspend beads prior to aliquoting them.

Note

C1 Streptavidin beads are preferred compared to M280 beads because they have higher binding capacity and use a negatively charged matrix. This significantly reduces carryover of non-biotinylated RNAs including adapter dimers.

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

19 Wash beads twice with 1 mL binding buffer.

Note

Always quickly spin samples down using a picofuge to remove liquid from tube caps between washes.

20 Resuspend the beads in 25 µL binding buffer per sample. Place beads On ice or at 4 °C until needed.

Run-On Reaction

30m

21 Prepare 2XROMM equilibrate at 37 °C (30 °C for Drosophila).

Note

When preparing the 2XROMM, first add all components other than Sarkosyl and mix by vortexing on high for >10 sec. Collect the solution with a quick spin, add Sarkosyl, and mix thoroughly by pipetting carefully to avoid bubbles. If you leave the 2XROMM on ice, a precipitate can form. Before use, check if this has occurred. The precipitate can be re-dissolved by heating at 37 °C for ~5 min and pipette mixing.

21.1 **2X Run-On Master Mix (2XROMM):**

[M] 10 millimolar (mM) Tris-Cl, pH 8.0

[M] 5 millimolar (mM) $MgCl_2$

[M] 1 millimolar (mM) DTT

[M] 300 millimolar (mM) KCl

[M] 40 micromolar (µM) Biotin-11-CTP

[M] 40 micromolar (µM) Biotin-11-UTP

[M] 40 micromolar (µM) Biotin-11-ATP

[M] 40 micromolar (µM) Biotin-11-GTP

[M] 1 Mass Percent Sarkosyl

in DEPC-treated ddH_2O

Add 1 µL SUPERase-In RNase Inhibitor per reaction.

Note

The run-on reaction uses 4 biotin-NTPs. However, ATP and GTP can be substituted at equal concentration for Biotin-11-ATP and Biotin-11-GTP to reduce cost. Biotin-11-ATP and biotin-11-GTP are 10X as expensive as biotin-11-CTP and biotin-11-UTP. With two biotin-NTPs blocking elongation, each polymerase can be expected to extend ~5 nt or less which we find gives sufficient resolution for the vast majority of applications. For low cell number experiments, increase the concentration of the biotin-NTPs to 500 µM. Biotin-NTP incorporation efficiency is ~60% with the concentration in the 2XROMM as written, which is sufficient for experiments using 10^6cells or greater, but increasing the concentration improves incorporation to ~77%.

22 Using a wide bore tip, add 50 µL of permeabilized cells to new 1.5 mL tube.

23 Pipette 50 µL of preheated 2XROMM into each reaction tube (already containing permeabilized cells). *Gently and thoroughly pipette the mixture 15 times*.

Safety information

It is extremely important to thoroughly mix the reaction so that nucleotides diffuse into highly viscous chromatin!

24 Incubate in a heat block or thermomixer at 37 °C (30 °C for Drosophila) at 750 RPM for 00:05:00 . Have RL buffer from Norgen kit or TRIzol LS ready for use.

25 Proceed to step 26.1 or 26.2 depending on choice of RNA extraction method immediately after the 00:05:00 reaction is complete (take the sample off the heat block and immediately add buffer RL or TRIzol LS).

Note

Stagger both addition of the 2XROMM and addition of TRIzol or buffer RL by 30 seconds or 1 minute, to ensure each sample is incubated exactly 5 min.

Total RNA extraction and Base Hydrolysis

1h 30m

26 Proceed from Step 25 to one of the following options:

Note

TRIzol LS or the Norgen Total RNA Purification Kit can be used to extract total RNA from the run-on reaction. Both options produce identical results. The Norgen kit is faster and less technically challenging to use, but more expensive. If TRIzol LS is used, Micro Bio-Spin™ RNase free P-30 Gel Columns are also needed to remove unincorporated biotin-NTPs as the biotin concentration will otherwise overwhelm the binding capacity of the streptavidin beads.

26.1 **Option 26.1: NORGEN RNA Extraction:**

1. Add 350 µL RL buffer and vortex.
2. Add 240 µL [M] 100 % (v/v) ethanol and vortex.
3. Apply solution to Norgen RNA extraction column.
4. Spin at 3,500 x g for 00:01:00 at 25 °C .
5. Add 400 µL wash solution A (ensure ethanol has been added).
6. Spin at 14,000 x g for 00:01:00 at 25 °C .
7. Discard flow through.
8. Repeat wash (steps 6 & 7) for a total of two washes.
9. Spin at 14,000 x g for 00:02:00 to dry column.
10. Add 50 µL DEPC-treated ddH_2O and vortex.

11. Elute by spinning at 200 x g for 00:02:00 at 25 °C and then at 14,000 x g for 00:01:00 at 25 °C .
12. Elute again with 50 µL DEPC-treated ddH_2O and pool eluates (V_f = 100 µL).
13. Denature at 65 °C for 00:00:30 and then snap cool on ice.
14. Add 25 µL ice cold [M] 1 Mass Percent NaOH and incubate 00:10:00 on ice.
15. Add 125 µL cold [M] 1 Mass Percent Tris-Cl pH 6.8, mix by pipetting.
16. Add 5 µL [M] 5 Mass Percent NaCl and 1 µL GlycoBlue and mix.
17. Add 625 µL [M] 100 % (v/v) Ethanol and vortex.

Note

If the protocol needs to be performed over two days, the ethanol precipitation in step 26.1.17 is the safest overnight stopping point. Store samples at -80 °C.

18. Centrifuge the samples at >20,000 x g for 00:20:00 at 4 °C .

Note

A blue pellet should be visible at the bottom of tube. The pellet can be difficult to see but should be visible. It may appear spread out. If a pellet is not visible, vortex well and repeat spin.

19. Carefully pipette supernatant off and discard.

Note

When removing the supernatant before the 70% ethanol wash be careful not to disturb the pellet. Approximately 30–50µL of ethanol can be left in the tube to avoid disturbing the pellet prior to adding the 70% ethanol wash. This procedure can also be used after the 70% ethanol wash (step 25.1.22), but then remove the final 30-50 µL using a P200 tip after a quick spin in a picofuge.

20. Add 750 µL [M] 70 % (v/v) ethanol.
21. Mix by gentle inversion and spin down briefly.
22. Carefully pipette supernatant off and discard.
23. Airdry the RNA pellet.

Note

Air dry the RNA pellet by leaving tubes open in fume hood to prevent contamination. This will take ~3-10 min depending on how much ethanol is left in the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its solubility.

24. Resuspend in 6 µL DEPC-treated ddH_2O.

26.2 **Option 26.2: Trizol LS RNA Extraction:**

1. Add 250 µL TRIzol LS with a wide bore P1000 tip and carefully pipette >10X until all white globs of nucleoproteins are homogenized.
2. Pipette mix again with a standard bore P1000 tip. Samples should be completely homogenous.
3. Vortex vigorously for at least 00:00:15 .
4. Incubate samples on ice until all run-on reactions are complete.
5. Add 65 µL chloroform.

Note

When pipetting chloroform, always pipette twice because the first draw always leaks.

6. Vortex the samples at max speed for 00:00:15 , then incubate on ice for 00:03:00 .
7. Centrifuge the samples at >20,000 x g for 00:08:00 at 4 °C .
8. Transfer the ~ 200 µL aqueous phase into a new tube.

Note

When transferring the aqueous phase of TRIzol extractions to a new tube, tilt the tube to a 45° angle and carefully remove only the clear liquid. Avoid contamination by the pink organic phase or white interphase.

9. Add 1 µL of GlycoBlue and mix.
10. Add 2.5X volumes (~ 500 µL) [M] 100 % (v/v) ethanol and vortex.
11. Centrifuge at > 20,000 x g for 00:20:00 at 4 °C .

Note

A blue pellet should be visible at the bottom of tube. The pellet can be difficult to see but should be visible. It may appear spread out. If a pellet is not visible, vortex well and repeat spin.

12. Carefully pipette supernatant off and discard.

Note

When removing the supernatant before the 70% ethanol wash be careful not to disturb the pellet. Approximately 30–50µL of ethanol can be left in the tube to avoid disturbing the pellet prior to adding the 70% ethanol wash. This procedure can also be used after the 70% ethanol wash (step 26.2.15), but then remove the final 30-50 µL using a P200 tip after a quick spin in a picofuge.

13. Add 750 µL [M] 70 % (v/v) ethanol.
14. Mix by gentle inversion and quickly spin down.
15. Carefully pipette supernatant off and discard.
16. Airdry the RNA pellet.

Note

Air dry the RNA pellet by leaving tubes open in fume hood to prevent contamination. This will take ~3-10 min depending on how much ethanol is left in the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its solubility.

17. Resuspend in 30 µL DEPC-treated ddH_2O.
18. Briefly denature at 65 °C for 00:00:30 and then snap cool On ice .
19. Add 7.5 µL ice cold [M] 1 Mass Percent NaOH and incubate On ice for 00:10:00 .
20. Add 37.5 µL [M] 1 Mass Percent Tris-Cl pH 6.8 , mix by pipetting.
21. Pass through a calibrated Bio-Rad RNase free P-30 column (follow manufacturer's instructions).
22. Bring volume to 200 µL with DEPC-treated ddH_2O (add ~ 125 µL).
23. Add 1 µL Glycoblue and 8 µL [M] 5 Mass Percent NaCl and vortex.
24. Add 500 µL [M] 100 % (v/v) ethanol and vortex.

Note

If the protocol needs to be performed over two days, the ethanol precipitation in 26.2.24 is the safest overnight stopping point. Store samples at -80 °C.

25. Centrifuge at >20,000 x g for 00:20:00 at 4 °C .

Note

A blue pellet should be visible at the bottom of tube. The pellet can be difficult to see but should be visible. It may appear spread out. If a pellet is not visible, vortex well and repeat spin.

26. Carefully pipette supernatant off and discard.

Note

When removing the supernatant before the 70% ethanol wash be careful not to disturb the pellet. Approximately 30–50µL of ethanol can be left in the tube to avoid disturbing the pellet prior to adding the 70% ethanol wash. This procedure can also be used after the 70% ethanol wash (step 26.2.29), but then remove the final 30-50 µL using a P200 tip after a quick spin in a picofuge.

27. Add 750 µL [M] 70 % (v/v) ethanol.
28. Mix by gentle inversion and quickly spin down.
29. Carefully pipette supernatant off and discard.
30. Airdry the RNA pellet.

Note

Air dry the RNA pellet by leaving tubes open in fume hood to prevent contamination. This will take ~3-10 min depending on how much ethanol is left in the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its solubility.

31. Resuspend in 6 µL DEPC-treated ddH_2O.

3'RNA Adapter Ligation

1h 15m

27 Continue here from step 26.1.24 or 26.2.31:

28 Add 1 µL [M] 10 micromolar (µM) VRA3 (V_f = 7 µL).

Note

The concentration of RNA adapters in the ligation steps (1 µL 10 µM) is optimal for approximately 10^6 mammalian cells. For lower cell numbers, the adapter concentration must be diluted to limit dimer formation. We dilute linearly with cell concentration relative to this established concentration, i.e. 1 µL 5 µM for 5 x 10^5 cells, 1 µL 2.5 µM for 2.5 x 10^5 cells, etc.

29 Denature at 65 °C for 00:00:30 and snap cool On ice .

30 Prepare ligation mix in the following order:

	Reagent	Volume
	10X T4 RNA Ligase Buffer	2 µL
	ATP (10 mM)	2 µL
	SUPERase-In RNase Inhibitor	1 µL
	50% PEG8000	6 µL
	T4 RNA LIgase 1 (ssRNA ligase)	2 µL

Note

Pipette slowly because 50% PEG8000 is very viscous. Heating 50% PEG8000 makes it easier to pipette. Pipette the ligation mix until it is homogenous before use.

Note

When preparing enzymatic reaction mixtures that contain SUPERase-In RNase Inhibitor, a fixed volume (1 µL) SUPERase-In can be added to the entire master mix regardless of number of reactions to decrease cost. Bring the remainder of the master mix up to the required volume with DEPC-treated ddH_2O. Murine RNase inhibitor can also be substituted to limit costs for all steps after the run-on. SUPERase-In is recommended prior to the run-on as it inhibits T1 RNase.

31 Add 13 µL and mix by pipetting 10–15X (V_f = 20 µL).

32 Incubate at 25 °C for 01:00:00 .

Streptavidin Bead Binding

45m

33 Add 55 µL binding buffer to each sample (V_f = 75 µL).

34 Add 25 µL pre-washed beads to each sample (V_f = 100 µL).

35 Incubate for 00:20:00 at 25 °C with end to end rotation.

36 Wash once with 500 µL High Salt Wash buffer with tube swap.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

For each washing step gently invert tubes 10–15X, quickly spin down with a picofuge, open caps, and then place on the magnet stand. Wait 1-2 minutes and pipette the supernatant off without disturbing the beads. If there are bubbles in the tube carefully pipette them off first and then remove supernatant. Beware that bubbles may dislodge beads from the side of the tube. After removing the bulk of the liquid, collect remaining liquid with a quick spin in a picofuge, place the tube back on the magnet stand, and carefully remove remaining liquid by pipetting.

Note

Transferring beads to a new tube after the binding incubation—during the high salt wash step—helps limit adapter dimer formation. After resuspending the beads in High Salt buffer, quickly spin down with a picofuge, resuspend beads by gently pipetting, and carefully transfer to a new tube. Pipette slowly to avoid bead loss! Place this new tube on the magnet stand and proceed with the washing protocol.

37 Wash once with 500 µL Low Salt Wash buffer.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

For each washing step gently invert tubes 10–15X, quickly spin down with a picofuge, open caps, and then place on the magnet stand. Wait 1-2 minutes and pipette the supernatant off without disturbing the beads. If there are bubbles in the tube carefully pipette them off first and then remove supernatant. Beware that bubbles may dislodge beads from the side of the tube. After removing the bulk of the liquid, collect remaining liquid with a quick spin in a picofuge, place the tube back on the magnet stand, and carefully remove remaining liquid by pipetting.

Note

Do not allow streptavidin beads to dry completely, as this can lead to clumping and make full resuspension impossible. When processing multiple samples, remove liquid from the previous wash or enzymatic step from the first sample and immediately resuspend those beads in the next solution, then repeat this process for additional samples.

On-Bead 5′ Hydroxyl Repair

45m

38 Resuspend beads in 19 µL PNK mix (V_f = 20 µL :

	Reagent	Volume
	DEPC-treated ddH2O	13 µL
	10X PNK buffer	2 µL
	10 mM ATP	2 µL
	T4 Polynucleotice Kinase	1 µL
	SUPERase-In RNase Inhibitor	1 µL

Note

On-bead reaction volumes assume that 1 µL of liquid remains on the beads.

Note

When preparing enzymatic reaction mixtures that contain SUPERase-In RNase Inhibitor, a fixed volume (1 µL) SUPERase-In can be added to the entire master mix regardless of number of reactions to decrease cost. Bring the remainder of the master mix up to the required volume with DEPC-treated ddH_2O. Murine RNase inhibitor can also be substituted to limit costs for all steps after the run-on. SUPERase-In is recommended prior to the run-on as it inhibits T1 RNase.

39 Incubate at 37 °C for 00:30:00 .

Note

Mix on-bead reactions by gently flicking the tubes every 10 minutes.

On-Bead 5′ Decapping

1h 15m

40 Place the tubes on a magnet stand and remove supernatant.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

Always quickly spin samples down using a picofuge to remove liquid from tube caps.

Note

Do not allow streptavidin beads to dry completely, as this can lead to clumping and make full resuspension impossible. When processing multiple samples, remove liquid from the previous wash or enzymatic step from the first sample and immediately resuspend those beads in the next solution, then repeat this process for additional samples.

41 Resuspend the beads in 19 µL RppH mix (V_f = 20 µL):

	Reagent	Volume
	DEPC-treated ddH2O	15 µL
	10X ThermoPol Buffer	2 µL
	RppH	1 µL
	SUPERase-In RNase Inhibitor	1 µL

Note

On-bead reaction volumes assume that 1 µL of liquid remains on the beads.

Note

We have also successfully used Cap-Clip™ Acid Pyrophosphatase (CELLTREAT) instead of RppH. Cap-Clip has lower buffer pH which may alleviate base hydrolysis of RNA that could occur in the pH 8.0 ThermoPol buffer. However, this is not a major concern except for in the most sensitive of applications.

Note

When preparing enzymatic reaction mixtures that contain SUPERase-In RNase Inhibitor, a fixed volume (1 µL) SUPERase-In can be added to the entire master mix regardless of number of reactions to decrease cost. Bring the remainder of the master mix up to the required volume with DEPC H_2O. Murine RNase inhibitor can also be substituted to limit costs for all steps after the run-on. SUPERase-In is recommended prior to the run-on as it inhibits T1 RNase.

42 Incubate at 37 °C for 01:00:00 .

Note

Mix on-bead reactions by gently flicking the tubes every 10 minutes.

On-Bead 5′ RNA Adaptor Ligation

1h 15m

43 Place the tubes on a magnet stand and remove supernatant.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

Always quickly spin samples down using a picofuge to remove liquid from tube caps.

Note

Do not allow streptavidin beads to dry completely, as this can lead to clumping and make full resuspension impossible. When processing multiple samples, remove liquid from the previous wash or enzymatic step from the first sample and immediately resuspend those beads in the next solution, then repeat this process for additional samples.

44

Resuspend the beads in 7 µL adapter mix (V_f = 8 µL):

	Reagent	Volume
	DEPC-treated ddH2O	6 µL
	REV5 (10 µM)	1 µl

Note

The concentration of RNA adapters in the ligation steps (1 µL 10 µM) is optimal for approximately 10^6 mammalian cells. For lower cell numbers, the adapter concentration must be diluted to limit dimer formation. We dilute linearly with cell concentration relative to this established concentration, i.e. 1 µL 5 µM for 5 x 10^5 cells, 1 µL 2.5 µM for 2.5 x 10^5 cells, etc.

45 Denature at 65 °C for 00:00:30, then snap cool On ice.

46 Prepare ligation mix in the following order:

	Reagent	Volume

	10X T4 RNA ligase buffer	2 µL
	ATP (10 mM)	2 µL
	SUPERase-In RNase Inhibitor	1 µL
	50% PEG8000	6 µL
	T4 RNA Ligase 1 (ssRNA ligase)	1 µL

Note

Pipette slowly because 50% PEG8000 is very viscous. Heating 50% PEG8000 makes it easier to pipette. Pipette the ligation mix until it is homogenous before use.

Note

Pipette slowly because 50% PEG8000 is very viscous. Heating 50% PEG8000 makes it easier to pipette. Pipette the ligation mix until it is homogenous before use.

Note

On-bead reaction volumes assume that 1 µL of liquid remains on the beads.

47 Add 12 µL to each tube (V_f = 20 µL).

48 Incubate at 25 °C for 01:00:00 .

Note

Mix on-bead reactions by gently flicking the tubes every 10 minutes.

TRIzol Elution of RNA

1h

49 Wash once with 500 µL High Salt Wash buffer with tube swap.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

For each washing step gently invert tubes 10–15X, quickly spin down with a picofuge, open caps, and then place on the magnet stand. Wait 1-2 minutes and pipette the supernatant off without disturbing the beads. If there are bubbles in the tube carefully pipette them off first and then remove supernatant. Beware that bubbles may dislodge beads from the side of the tube. After removing the bulk of the liquid, collect remaining liquid with a quick spin in a picofuge, place the tube back on the magnet stand, and carefully remove remaining liquid by pipetting.

Note

Transferring beads to a new tube after the binding incubation—during the high salt wash step—helps limit adapter dimer formation. After resuspending the beads in High Salt buffer, quickly spin down with a picofuge, resuspend beads by gently pipetting, and carefully transfer to a new tube. Pipette slowly to avoid bead loss! Place this new tube on the magnet stand and proceed with the washing protocol.

50 Wash once with 500 µL Low Salt Wash buffer.

Note

Be careful not to disturb beads when removing buffers from tubes. Open tube caps prior to placing them on the magnet stand, as opening on the magnet stand can disturb the liquid. Check pipette tip against a white background before discarding liquid to ensure beads are not present.

Note

For each washing step gently invert tubes 10–15X, quickly spin down with a picofuge, open caps, and then place on the magnet stand. Wait 1-2 minutes and pipette the supernatant off without disturbing the beads. If there are bubbles in the tube carefully pipette them off first and then remove supernatant. Beware that bubbles may dislodge beads from the side of the tube. After removing the bulk of the liquid, collect remaining liquid with a quick spin in a picofuge, place the tube back on the magnet stand, and carefully remove remaining liquid by pipetting.

Note

Do not allow streptavidin beads to dry completely, as this can lead to clumping and make full resuspension impossible. When processing multiple samples, remove liquid from the previous wash or enzymatic step from the first sample and immediately resuspend those beads in the next solution, then repeat this process for additional samples.

51 Resuspend beads in 300 µL TRIzol.

52 Vortex at max speed for > 00:00:20 , then incubate On ice for 00:03:00 .

53 Add 60 µL chloroform.

Note

When pipetting chloroform, always pipette twice because the first draw always leaks.

54 Vortex at max speed for 00:00:15 , then incubate On ice for 00:03:00 .

55 Centrifuge at > 20,000 x g for 00:08:00 at 4 °C .

56 Transfer the aqueous phase (~ 180 µL) to a new tube.

Note

When transferring the aqueous phase of TRIzol extractions to a new tube, tilt the tube to a 45° angle and carefully remove only the clear liquid. Avoid contamination by the pink organic phase or white interphase.

57 Add 1 µL GlycoBlue and mix.

58 Add 2.5X volumes (~ 450 µL) [M] 100 % (v/v) ethanol and vortex.

59 Centrifuge the samples at > 20,000 x g for 00:20:00 at 4 °C .

Note

A blue pellet should be visible at the bottom of tube. The pellet can be difficult to see but should be visible. It may appear spread out. If a pellet is not visible, vortex well and repeat spin.

60 Carefully pipette supernatant off and discard.

Note

When removing the supernatant before the 70% ethanol wash be careful not to disturb the pellet. Approximately 30–50µL of ethanol can be left in the tube to avoid disturbing the pellet prior to adding the 70% ethanol wash. This procedure can also be used after the 70% ethanol wash (step 63), but then remove the final 30-50 µL using a P200 tip after a quick spin in a picofuge.

61 Add 750 µL [M] 70 % (v/v) ethanol.

62 Mix by gentle inversion and quickly spin down.

63 Carefully pipette supernatant off and discard.

64 Airdry the RNA pellet.

Note

Air dry the RNA pellet by leaving tubes open in fume hood to prevent contamination. This will take ~3-10 min depending on how much ethanol is left in the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its solubility.

Off-Bead Reverse Transcription

1h 15m

65 Resuspend RNA pellet in 13.5 µL RT resuspension mix:

	Reagent	Volume
	DEPC-treated ddH2O	8.7 µL
	Primer RP1 (10 µM)	4 µL
	dNTP mix (12.5 mM each)	0.8 µL

Note

Reverse transcription can also be performed on-bead, but we find that this significantly reduces library yield while increasing adapter dimer. For this reason, it is not recommended except in cases where material is abundant (10^7 cells) and speed is paramount. To do this, follow steps 49 and 50, then skip to step 65, but resuspend the beads instead of the RNA pellet in RT resuspension mix. After RT, elute cDNA by heating the bead mixture to 95°C, quickly place tubes on a magnet stand, and remove and save supernatant. Resuspend beads in 20 µL ddH_2O and repeat the process for a final volume of 40 µL. Proceed with PreCR but use 20 µL less ddH_2O (13.5 µL) in the PreCR mix and use the entire 40 µL eluate instead of the 20 µL RT mix.

66 1.Denature at 65 °C for 00:05:00 and snap cool On ice .

67 1.Prepare RT master mix:

	Reagent	Volume
	5X RT Buffer	4 µL
	100 mM DTT	1 µL

	SUPERase-In RNase Inhibitor	0.5 µL
	Maxima H Minus RT enzyme	1 µL

68 Add 6.5 µL to each sample (V_f = 20 µL).

69 Cycle as follows:

a. 50 °C for 00:30:00

b. 65 °C for 00:15:00

c. 85 °C for 00:05:00

d. hold at 4 °C.

70 Immediately proceed to PreCR, test amplification, or full-scale amplification. Samples can be stored overnight at -20 °C (see Notes 38–39).

Note

PreCR is optional if full scale amplification will be performed within 2 days. Longer storage of single-stranded cDNA libraries can lead to loss of library material. If you are skipping PreCR, simply store the 20 µL RT reaction at -20°C overnight and perform test amplification the next day.

Note

Because this protocol uses molecular barcodes (UMIs) which facilitate robust computational PCR deduplication, it is less important to precisely determine the optimal cycle number. We recommend performing test amplification the first time you perform this protocol with a given amount of material from a given cell line to determine the optimal cycle number. For future experiments where the material and cell number are constant, test amplification can be skipped. Adjust the volume of the full-scale PCR to 100 µL total volume (accounting for the fact that the written protocol assumes loss due to test amplification). Test amplification can be performed either by PCR of a dilution curve and PAGE analysis or qPCR.

PreCR

1h 30m

71

Note

Because this protocol uses molecular barcodes (UMIs) which facilitate robust computational PCR deduplication, it is less important to precisely determine the optimal cycle number. We recommend performing test amplification the first time you perform this protocol with a given amount of material from a given cell line to determine the optimal cycle number. For future experiments where the material and cell number are constant, test amplification can be skipped. Adjust the volume of the full-scale PCR to 100 µL total volume (accounting for the fact that the written protocol assumes loss due to test amplification). Test amplification can be performed either by PCR of a dilution curve and PAGE analysis or qPCR.

72 Add 2.5 µL RPI-n indexed primer ([M] 10 micromolar (µM)) to each sample. Use different barcodes for samples that will be pooled and sequenced together.

73 Prepare the PreCR master mix:

	Reagent	Volume
	ddH2O	33.5 µL
	5X Q5 Buffer	20 µL
	5X Q5 Enhancer	20 µL
	Primer RP1 (10 µM)	1 µL
	dNTP mix (12.5 mM each)	2 µL
	Q5 Polymerase	1 µL

74 Add 77.5 µL of the PreCR mix to each sample for final volume 100 µL .

Note

Do not attempt to scale down the PreCR or full-scale amplification steps to save PCR reagents. If RT reaction mixture exceeds 20% of the PCR reaction volume, significant inhibition of PCR will occur and lead to dramatically lower final library yield.

75 Amplify libraries for 5 cycles on thermal cycler using the following settings:

a. 95 °C for 00:02:00

b. 95 °C for 00:00:30

c. 56 °C for 00:00:30

d. 72 °C for 00:00:30

e. Go to b. 4 more times

f. 72 °C for 00:05:00

g. Hold at 4 °C

76 Store samples at -20 °C or proceed to test amplification.

Test Amplification (Gel)

4h

77

Note

Because this protocol uses molecular barcodes (UMIs) which facilitate robust computational PCR deduplication, it is less important to precisely determine the optimal cycle number. We recommend performing test amplification the first time you perform this protocol with a given amount of material from a given cell line to determine the optimal cycle number. For future experiments where the material and cell number are constant, test amplification can be skipped. Adjust the volume of the full-scale PCR to 100 µL total volume (accounting for the fact that the written protocol assumes loss due to test amplification). Test amplification can be performed either by PCR of a dilution curve and PAGE analysis or qPCR

Note

Taking 7.7 µL of the 100 µL PreCR reaction leaves 92.3 µL for full-scale amplification. 25% of material in the first dilution is lost to make the next serial 4-fold dilution (2 of 8 µL). Because (7.7 * 0.75) / 92.3 ≈ 1/16, this first dilution is equivalent to the number of test amplification cycles less 4. If starting from the RT reaction, the volume has been adjusted for 5-fold lower starting volume.

Make the first dilution using one of the following options:

77.1 If PreCR was performed, add 7.7 µL of the 100 µL PreCR reaction to 0.3 µL ddH_2O for a final volume of 8 µL .

77.2 If PreCR was skipped, add 1.54 µL of the 20 µL RT reaction to 6.46 µL ddH_2O for a final volume of 8 µL.

78 Make 4-fold serial dilutions by adding 2 µL of each dilution to 6 µL ddH_2O for the next dilution.

79 Remove and discard 2 µL from the final dilution (all dilutions should now be 6 µL).

80 Choose a target number of total cycles for test amplification using the table below (see Note 42). The first dilution simulates full-scale amplification at the total number of cycles (PreCR cycles + Test Amp cycles) minus 4. Subtract 2 cycles sequentially for the following dilutions.

	Dilution	1	2	3	4	5	6	7	8
	19 Total Cycles	15	13	11	9	7	5	3	1
	21 Total Cycles	17	15	13	11	9	7	5	3
	23 Total Cycles	19	17	15	13	11	9	7	5

Note

Additional cycles can vary by cell type. For HeLa, we typically perform 14 additional cycles (19 total cycles), which simulates 15 full-scale amplification cycles. For low input libraries (50k-250k mammalian cells), we typically perform 20 additional cycles (23 cycles total), which simulates 21 full-scale amplification cycles.

81 Make test PCR mix:

	Reagent	Volume
	ddH2O	4.4 µL
	5X Q5 Buffer	4 µL
	5X Q5 Enhancer	4 µL

	Primer RP1 (10 µM)	0.5 µL
	Primer RPI-n (10 µM)	0.5 µL
	dNTP mix (12.5 mM each)	0.4 µL
	Q5 Polymerase	0.2 µL

82 Add 14 µL PCR mix to the 6 µL diluted test samples (V_f = 20 µL).

83 Amplify reactions for the desired amount of cycles using following settings:

Safety information

CRITICAL Remember to account for PreCR. Subtract 5 cycles from your total target test amplification cycles.

a. 95 °C for 00:02:00
b. 95 °C for 00:00:30
c. 65 °C for 00:00:30
d. 72 °C for 00:00:30
e. Go to step 2 for the desired number of cycles
f. 72 °C for 00:05:00
g. Hold at 4 °C

Note

If PreCR was skipped, use an annealing temperature of 56°C for the first 5 cycles of test amplification and the full-scale amplification.

84 Mix with gel loading dye to 1X and run 10 µL on a [M] 2.2 Mass Percent Agarose gel or run 2 µL on a native [M] 8 % (v/v) polyacrylamide gel and stain with SYBR Gold.

85 Use the test amplification gel to determine the appropriate number of cycles for full-scale amplification.

Note

Desired amplification characteristics include a sufficient amount of product (smear starting ~150 bp), no evidence of overamplification, and ~50% primer exhaustion. The adaptor dimer product is 132 bp, and the smear will start 15–20 bp above this band. RNA degradation will lead to shorter library products. See:

Citation

Mahat DB, Kwak H, Booth GT, Jonkers IH, Danko CG, Patel RK, Waters CT, Munson K, Core LJ, Lis JT
(Invalid date)
. Base-pair-resolution genome-wide mapping of active RNA polymerases using precision nuclear run-on (PRO-seq).
Nature protocols.

https://doi.org/10.1038/nprot.2016.086 LINK

3.14 Test Amplification (qPCR) 4h

86

Note

Because this protocol uses molecular barcodes (UMIs) which facilitate robust computational PCR deduplication, it is less important to precisely determine the optimal cycle number. We recommend performing test amplification the first time you perform this protocol with a given amount of material from a given cell line to determine the optimal cycle number. For future experiments where the material and cell number are constant, test amplification can be skipped. Adjust the volume of the full-scale PCR to 100 µL total volume (accounting for the fact that the written protocol assumes loss due to test amplification). Test amplification can be performed either by PCR of a dilution curve and PAGE analysis or qPCR

Add 1.54 µL of the 20 µL RT reaction to 0.46 µL ddH_2O (V_f = 2 µL).

87 Make the qPCR master mix:

	Reagent	Volume
	Primer RP1 (10 µM)	0.25 µL
	Primer RPI-n (10 µM)	0.25 µL
	2X SsoAdvanced Universal SYBR Green Supermix	5 µL
	ddH2O	2.5 µL

88 Add 8 µL of the qPCR master mix to 2 µL diluted RT reaction (V_f = 10 µL).

89 Quickly spin plate to collect liquid.

90 Amplify in a real-time PCR system using the following conditions:

90.1 **Amplification**

a. 98 °C for 00:02:00

b. 98 °C for 00:00:15

c. 60 °C for 00:01:00

d. Go to step 2 for 39 additional cycles

90.2 **Melt Curve**

a. 95 °C for 00:00:15

b. 60 °C for 00:01:00

c. 96 °C for 00:00:15

d. 60 °C for 00:00:16

91 Calculate the number of full-scale amplification cycles as the cycle number where Rn reaches 0.25 × Rn_{max}.

Full-Scale Amplification

1h 30m

92 **If PreCR and Test Amplification were skipped:**

92.1 Add 2.5 µL of an RPI-n indexed primer ([M] 10 micromolar (µM)) to each 20 µL RT reaction. Use different barcodes for samples that will be pooled and sequenced on a single lane.

92.2 Prepare the PCR master mix:

	Reagent	Volume
	ddH2O	33.5 µL
	5X Q5 Buffer	20 µL
	5X Q5 Enhancer	20 µL
	Primer RP1 (10 µM)	1 µL
	dNTP mix (12.5 mM each)	2 µL
	Q5 Polymerase	1 µL

Note

Do not attempt to scale down the PreCR or full-scale amplification steps to save PCR reagents. If RT reaction mixture exceeds 20% of the PCR reaction volume, significant inhibition of PCR will occur and lead to dramatically lower final library yield.

92.3 Add 77.5 µL PCR master mix to each sample for final volume 100 µL .

92.4 Run the desired number of cycles:

a. 95 °C for 00:02:00

b. 95 °C for 00:00:30

c. 56 °C for 00:00:30
d. 72 °C for 00:00:30
e. Go to step (b) for 4 more cycles.
f. 95 °C for 00:00:30
g. 65 °C for 00:00:30
h. 72 °C for 00:00:30
i. Go to step (f) for the desired number of cycles
j. Hold at 4 °C

93 **If PreCR was skipped but Test Amplification was performed:**

93.1 Add 2.5 µL of an RPI-n indexed primer ([M] 10 micromolar (µM)) to the remaining 18.5 µL RT reaction. Use different barcodes for samples that will be pooled and sequenced on a single lane.

93.2 Prepare the PCR master mix:

	Reagent	Volume
	ddH2O	35 µL
	5X Q5 Buffer	20 µL
	5X Q5 Enhancer	20 µL
	Primer RP1 (10 µM)	1 µL
	dNTP mix (12.5 mM each)	2 µL
	Q5 Polymerase	1 µL

Note

Do not attempt to scale down the PreCR or full-scale amplification steps to save PCR reagents. If RT reaction mixture exceeds 20% of the PCR reaction volume, significant inhibition of PCR will occur and lead to dramatically lower final library yield.

93.3 Add 79 µL PCR master mix to each sample for final volume 100 µL .

93.4 Run the desired number of cycles:

a. 95 °C for 00:02:00

b. 95 °C for 00:00:30

c. 56 °C for 00:00:30

d. 72 °C for 00:00:30

e. Go to step (b) for 4 more cycles.

f. 95 °C for 00:00:30

g. 65 °C for 00:00:30

h. 72 °C for 00:00:30

i. Go to step (f) for the desired number of cycles

j. Hold at 4 °C

94 **If PreCR and Test Amplification were performed:**

94.1 Prepare the following spike-in PCR mix:

	Reagent	Volume
	ddH2O	3.7 µL
	5X Q5 Buffer	1.5 µL
	5X Q5 Enhancer	1.5 µL
	dNTP mix (12.5 mM)	0.5 µL
	Q5 Polymerase	0.5 µL

94.2 Add 7.7 µL PCR spike-in mix to each sample for final volume 100 µL .

94.3 Run the desired number of cycles.

a. 95 °C for 00:02:00

b. 95 °C for 00:00:30

c. 65 °C for 00:00:30

d. 72 °C for 00:00:30

e. Go to step (b) for the desired number of cycles

f. Hold at 4 °C

Safety information

Remember to account for PreCR. Subtract 5 cycles from your total target full-scale amplification cycles.

95 Allow PCR reactions to reach room temperature.

96 Add 180 µL SPRI beads (see Note 45) at room temperature and immediately mix by pipetting > 15X.

97 Incubate at Room temperature for 00:05:00 .

98 Place on a magnet stand and remove the supernatant.

99 Wash the beads twice with [M] 70 % (v/v) ethanol without resuspending.

Safety information

Do not disturb the beads or library recovery will be greatly reduced.

100 Airdry the beads for 00:05:00 . Do not over dry the beads.

101 Resuspend beads in 22 µL [M] 10 millimolar (mM) Tris-Cl, pH 8.0 (no EDTA).

102 Incubate at room temperature for 00:05:00 .

103 Place the beads on a magnet stand and transfer 20 µL to a new tube.

104 Quantify the library using the Qubit dsDNA-HS assay and run on a Bioanalyzer.

PAGE purification

1d

105

Note

Due to advances in streptavidin bead technology and titration of adapters presented in this protocol, PAGE purification is rarely necessary. We prefer to sequence libraries that are 0%–25% adapter dimer rather than risk size bias associated with gel purification. Only perform PAGE purification if absolutely necessary. If needed, multiple libraries can be pooled by molarity as determined by bioanalyzer and extracted from the same gel lane to minimize size bias.

106 Add Orange G loading dye to 1X to the entire library volume.

107 Run the samples on a native [M] 8 % (v/v) polyacrylamide gel.

108 Stain with SYBR Gold.

109 Cut a gel slice from immediately above the adapter dimer to ~650 bp.

Note

Desired amplification characteristics include a sufficient amount of product (smear starting ~150 bp), no evidence of overamplification, and ~50% primer exhaustion. The adaptor dimer product is 132 bp, and the smear will start 15–20 bp above this band. RNA degradation will lead to shorter library products. See:

Citation

Mahat DB, Kwak H, Booth GT, Jonkers IH, Danko CG, Patel RK, Waters CT, Munson K, Core LJ, Lis JT
(Invalid date)
. Base-pair-resolution genome-wide mapping of active RNA polymerases using precision nuclear run-on (PRO-seq).
Nature protocols.

https://doi.org/10.1038/nprot.2016.086 LINK

110 Place the gel slice in a 0.5 mL microfuge tube.

111 Make a hole in the bottom of the tube with an 18G needle.

112 Nest the 0.5 mL tube in a 1.5 mL tube and spin at 5000 x g for 00:01:00 .

113 If gel remains in the 0.5 mL tube, repeat step 7 and pool shredded gel fractions by suspending each in 250 µL soaking buffer using a wide-bore P1000 tip.

114 Soak the gel pieces in 0.5 mL soaking buffer (TE + [M] 150 millimolar (mM) NaCl + [M] 0.1 % (v/v) Tween-20) overnight with agitation at 37 °C .

115 Spin the tube at 5000 x g for 1 min.

116 Pipette as much of the soaking buffer as possible without transferring gel pieces into a new tube.

117 Add an additional 0.5 mL soaking buffer and incubate 04:00:00 at 37 °C with agitation.

118 Spin the tube at 5000 x g for 00:01:00 .

119 Pipette as much of the soaking buffer as possible without transferring gel pieces into the tube with the previous eluate.

120 Pass the remaining gel solution through a Costar Spin-X column using a cut P1000 tip and pool with the previous eluate (V_f = 1 mL)

121 Reduce the volume by half (V_f = 0.5 mL) using vacuum dryer at 37 °C .

122 Add 1 µL GlycoBlue.

123 Add 2.5X volume (1.25 mL) [M] 100 % (v/v) ethanol and vortex.

124 Centrifuge at >20,000 x g for 00:20:00 at 4 °C .

> Note
>
> A blue pellet should be visible at the bottom of tube. The pellet can be difficult to see but should be visible. It may appear spread out. If a pellet is not visible, vortex well and repeat spin.

125 Carefully pipette off the supernatant and discard.

Note

When removing the supernatant before the 70% ethanol wash be careful not to disturb the pellet. Approximately 30–50µL of ethanol can be left in the tube to avoid disturbing the pellet prior to adding the 70% ethanol wash. This procedure can also be used after the 70% ethanol wash, but then remove the final 30-50 µL using a P200 tip after a quick spin in a picofuge.

126 Add 750 µL of [M] 75 % (v/v) ethanol.

127 Mix by gentle inversion and quickly spin down.

128 Carefully pipette off the supernatant and discard.

129 Air-dry the RNA pellet.

Note

Air dry the RNA pellet by leaving tubes open in fume hood to prevent contamination. This will take ~3-10 min depending on how much ethanol is left in the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its solubility.

130 Resuspend the pellet in the desired volume of [M] 10 millimolar (mM) Tris-Cl, pH 8.0, no EDTA

Computational Analysis

131 A pipeline for alignment of PRO-seq data can be found here: https://github.com/JAJ256/PROseq_alignment.sh