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1 # A rapid, sensitive, scalable method for
2 # Precision Run-On sequencing (PRO-seq)
3

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23 **Abstract**

24

25 Tracking active transcription with the nuclear run-on (NRO) assays has been instrumental in
26 uncovering mechanisms of gene regulation. The coupling of NROs with high-throughput
27 sequencing has facilitated the discovery of previously unannotated or undetectable RNA classes
28 genome-wide. Precision run-on sequencing (PRO-seq) is a run-on variant that maps polymerase
29 active sites with nucleotide or near-nucleotide resolution. One main drawback to this and many
30 other nascent RNA detection methods is the somewhat intimidating multi-day workflow
31 associated with creating the libraries suitable for high-throughput sequencing. Here, we present
32 an improved PRO-seq protocol where many of the enzymatic steps are carried out while the
33 biotinylated NRO RNA remains bound to streptavidin-coated magnetic beads. These
34 adaptations reduce time, sample loss and RNA degradation, and we demonstrate that the
35 resulting libraries are of the same quality as libraries generated using the original published
36 protocol. The assay is also more sensitive which permits reproducible, high-quality libraries from
37 10^4–10^5 cells instead of 10^6–10^7. Altogether, the improved protocol is more tractable allows for
38 nascent RNA profiling from small samples, such as rare samples or FACS sorted cell
39 populations.

40

41 **Keywords:** Nuclear run-on, Nascent RNA, Transcription, RNA polymerase, non-coding RNA

42

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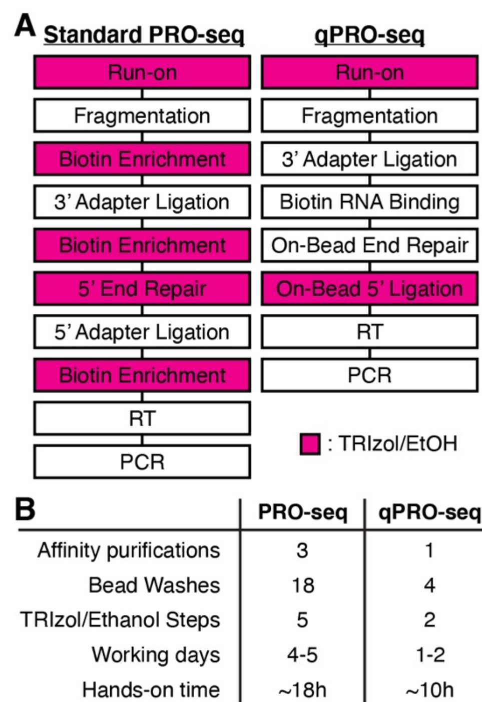
1. Introduction

Next-generation sequencing technologies are now routinely used to measure gene expression levels in a highly sensitive and comprehensive fashion. RNA-seq facilitate simultaneous detection, identification, and annotation of many classes of cellular RNAs. Traditionally, however, this technology primarily measures steady-state RNA levels, which are a consequence of equilibrium between RNA transcription, processing, and degradation. As a result, many unstable RNAs, particularly eRNA and some lncRNAs, are not easily detected with these approaches. Alternatively, ChIP-seq allows for identification and quantification of RNA polymerase II (Pol II) associated DNA. This produces a genome-wide map of both transcriptionally active and inactive Pol II without strand specificity, thus the direction and transcriptional status of the polymerase are ambiguous. Furthermore, the relatively high background in ChIP-seq as compared to RNA-based methods obfuscates comprehensive transcript and regulatory element detection. To address these shortcomings, various methods of measuring transcription by genome-wide profiling of nascent RNA have now been developed including GRO-seq (Core et al., 2008), PRO-seq (Kwak et al., 2013), NET-seq (Churchman and Weissman, 2011), and TT-seq (Schwalb et al., 2016). Characteristics of these assays are reviewed in (Wissink et al., 2019).



	PRO-seq	qPRO-seq
Affinity purifications	3	1
Bead Washes	18	4
TRIzol/Ethanol Steps	5	2
Working days	4-5	1-2
Hands-on time	~18h	~10h

Figure 1: A rapid and efficient PRO-seq protocol. (A) Overview of changes to the PRO-seq protocol implemented in qPRO-seq. **(B)** Comparison of steps and time required to complete each protocol.

GRO-seq and PRO-seq are modern, genome-wide improvements of the nuclear run-on assay, which has been in use for approximately 60 years (Weiss and Gladstone, 1959). Over the years, run-on assays have been instrumental in the study or discovery of various forms of gene regulation including, steady state transcription levels and mRNA turnover (Derman et al., 1981; Powell et al., 1984), promoter-proximal pausing (Gariglio et al., 1981; Rougvie and Lis, 1988), transcription rates (Bentley and Groudine, 1986; Hirayoshi and Lis, 1999; O'Brien and Lis, 1993), and 3'-end processing and termination (Birse et al., 1997). The nuclear run-on reaction works by adding labelled nucleotides to polymerases that are halted in the act of transcription yet still transcriptionally competent. The polymerases incorporate the exogenous NTPs and the labelled nascent transcripts can then be detected and quantified. In 2004, the assay was adapted for macro-array by spotting probes of whole yeast genes on to nylon membranes (García-Martínez et al., 2004). In 2008, the nuclear run-on assay was expanded to cover the entire genome in global run-on and sequencing (GRO-seq) (Core et al., 2008). GRO-

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seq employs a BrU-containing run-on reaction, enabling affinity purification and subsequent high-throughput sequencing of the entire nascent transcriptome. This allows sensitive and strand specific measurement of all transcriptionally engaged RNA polymerases, but resolution is limited because run-on length can only be loosely controlled via limiting nucleotide titration.

To address this shortcoming, precision nuclear run-on and sequencing (PRO-seq) uses a similar run-on and sequencing strategy but substitutes bulky, biotin-tagged NTPs instead of BrU (Kwak et al., 2013; Mahat et al., 2016). Biotin effectively halts eukaryotic RNA polymerases after incorporation of a single NTP, which enables genome-scale profiling of transcriptionally engaged polymerases with base-pair precision. PRO-seq and the associated technology ChRO-seq (Chu et al., 2018; Kwak, 2013) have now been used to study the transcriptional kinetics of the heat-shock response in human (Vihervaara et al., 2017), mouse (Mahat et al., 2016), and fly (Duarte et al., 2016), for *de novo* discovery of promoters and enhancers in cells and tissues (Chu et al., 2018; Danko et al., 2015; Kruesi et al., 2013; Kwak, 2013; Wang et al., 2019), for transcription rate detection (Danko et al., 2013; Jonkers et al., 2014), and detection of RNA stability (Blumberg et al., 2019; Core et al., 2014).

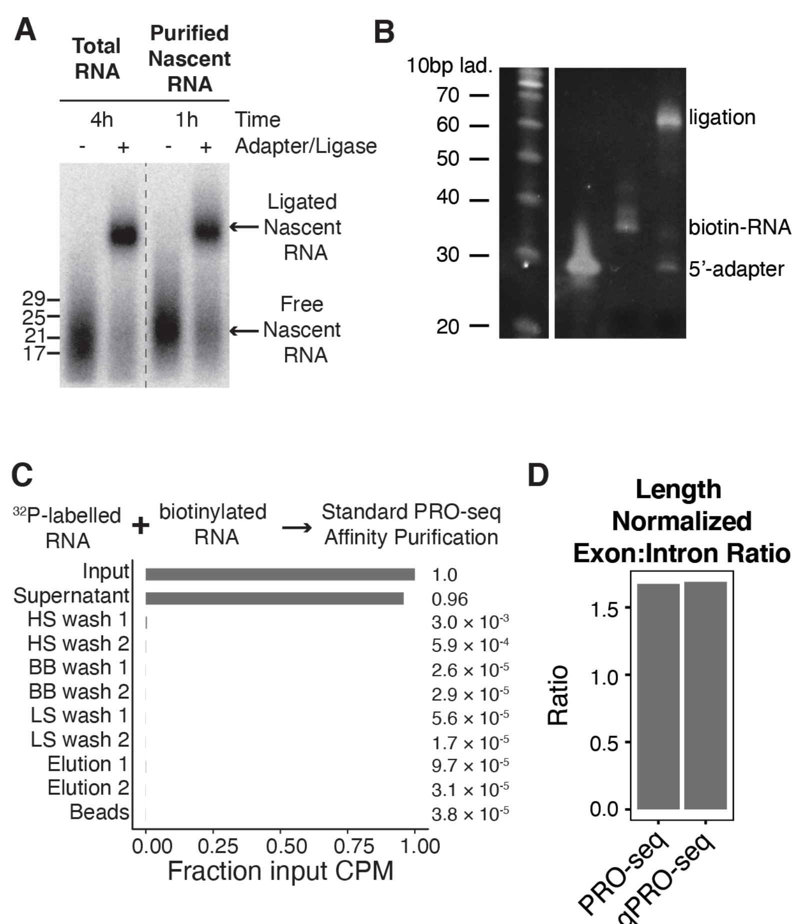

Figure 2: Validation of key enzymatic steps. **(A)** Ligation of the 3' adapter in hydrolyzed total RNA vs. purified nascent RNA. ^{32}P labelled nascent RNA was ligated for 1 h using the ligation conditions in this protocol or using the standard PRO-seq ligation conditions in (Mahat et al., 2016). **(B)** Efficiency of ligation to synthetic biotinylated RNA in 15% PEG8000 **(C)** Stringency of biotin-RNA affinity purification with MyOne C1 Streptavidin beads. Excess ^{32}P labelled non-biotinylated RNA was mixed with biotinylated RNA, and CPM of each fraction was assessed using liquid scintillation counting. **(D)** PRO-seq and qPRO-seq show similar levels of exonic reads relative to intronic reads genome-wide.

The current, published PRO-seq protocol (Mahat et al., 2016) is undoubtedly time consuming, technically challenging, and requires significant amounts of starting material (0.5–2 x 10^7 cells). This is largely due to multiple streptavidin bead binding and subsequent elution steps (Fig. 1A), which require technical finesse with phenol:chloroform extraction and ethanol

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119 precipitation of nucleic acids and present repeated opportunity for loss of material. This has
120 limited the adoption of PRO-seq by inexperienced laboratories and impeded studies of
121 transcription in experimental systems that utilize rare or precious biological samples.
122
123 To address these shortcomings, we optimized the PRO-seq protocol to simplify library
124 preparation and facilitate use of scarce input material in an improved protocol deemed qPRO-
125 seq (quick Precision Run-On and sequencing; Fig. 1). The original protocol requires 4-5 working
126 days to complete, and included three bead binding steps, and five phenol:chlofororm extractions
127 and ethanol precipitations. By performing 3' adapter ligation to hydrolyzed total RNA instead of
128 affinity purified nascent RNA, we eliminated one bead-binding step. We have validated that this
129 ligation reaction is equally efficient to the standard PRO-seq ligation to purified nascent RNA
130 (Fig. 2A). Downstream enzymatic reactions are then performed while nascent RNA is affixed to
131 streptavidin beads (Fig. 2B), which eliminates another bead-binding step. On-bead reactions are
132 performed in 2X volume to aid in handling, and simple bead washing steps replace numerous
133 phenol:chloroform extractions and ethanol precipitations. The resulting single bead-binding
134 protocol is much faster and easier than the original PRO-seq protocol (Fig. 1). It is feasible to
135 start from permeabilized cells and end with adapter ligated cDNA in a single day (~10 h; Fig.
136 1B). Furthermore, an option for column-based purification of RNA after the run-on reaction can
137 eliminate another organic extraction step. Reverse transcription can be performed on beads,
138 which completely eliminates organic extraction from the protocol, albeit with reduced efficiency.
139
140 In theory, reducing the number of affinity purifications and ligating adapters to bulk RNAs
141 could reduce the specificity of the assay for nascent RNA. However, we have found that MyOne
142 Streptavidin C1 beads, which have higher binding capacity per substrate area and are negatively
143 charged to repel non-specific nucleic acid binding, sufficiently enrich nascent RNA over other
144 cellular RNAs (Fig. 2C). Importantly, if nascent RNA was contaminated with mRNA, the number
145 of reads mapping within exons would increase relative to reads mapping within introns. However,
146 when we compared the length-normalized ratio of exonic reads to intronic reads, we observed
147 no detectable difference between the original protocol and the improved protocol presented here
148 (Fig. 2D).
149
150 Additional protocol changes have further simplified and improved the protocol. Careful
151 titration of adapters eliminates the need for PAGE purification (Fig. 3A–B), which is difficult and
152 time intensive and can introduce insert-size bias in the final libraries. Incorporation of a dual-UMI
153 strategy reduces concern about PCR-duplicate reads. Furthermore, this eliminates the need for
154 time-consuming test-amplification, except as an initial troubleshooting step when establishing
155 the assay in a new cell type or with a new amount of input material.
156

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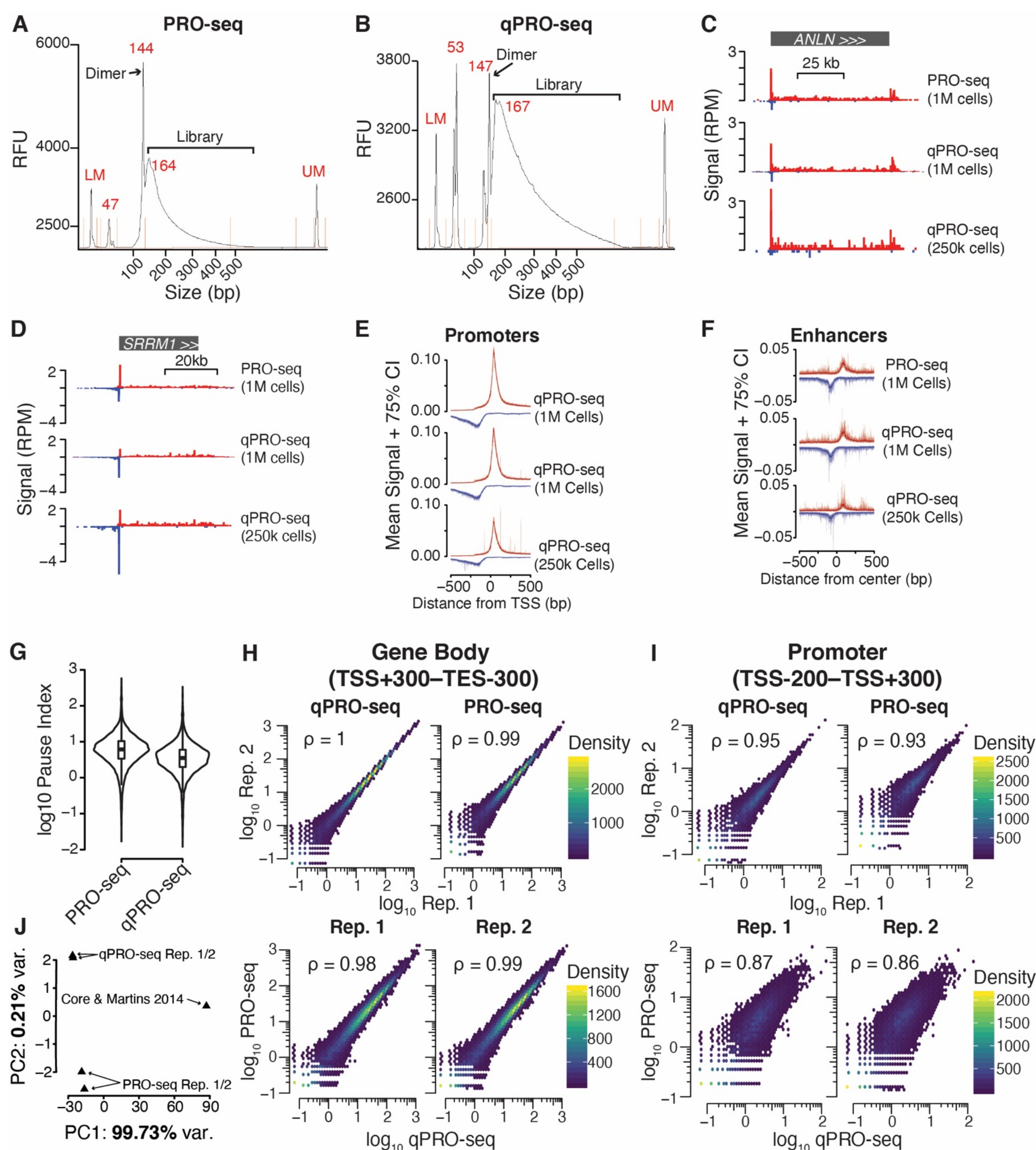

157

Figure 3: Direct comparison of PRO-seq and qPRO-seq in K562 cells. Bioanalyzer traces of libraries made using PRO-seq **(A)** and qPRO-seq. Library and adapter dimer are highlighed. **(B).** Browser shots showing reads-per-million (RPM) normalized signal for PRO-seq (1M cells), qPRO-seq (1M cells), and qPRO-seq (250k cells) at *ANLN* **(C)** and *SRRM1* **(D).** Metaprofiles of signal at promoters **(E)** and enhancers **(F).** Mean and 75% confidence intervals are derived from 1000 subsamplings of 10% of regions. Enhancers are dREG peaks > 10 kb from a promoter. **(G)** Distribution of pause indicies at all genes in PRO-seq compared to qPRO-seq. **(H)** Correlation of RPM in gene body regions (TSS+300 to TES-300) between replicates and between protocols. **(I)** Correlation of RPM in promoter regions (TSS-200 to TSS+300) between replicates and between protocols. **(J)** Principal component analysis of PRO-seq, qPRO-seq, and published PRO-seq (Core et al. 2014) data in K562 cells. Variance explained (%) by each component is noted on the axis.

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158 We have found that 10^6 cells are sufficient input material for this new protocol, and that it can
159 even be performed with as few as 0.05–0.25 x 10^6 cells (Fig 3C–F). Data quality typically
160 increases up to 10^6 cells but much smaller improvements are seen by further increasing cell
161 number. Polymerases are sampled from fewer positions overall in libraries made with 0.25 x 10^6
162 cells, which causes data to look “spiky” and inflates read counts at highly expressed genes when
163 normalizing per million mapped reads (Fig. 3C–F). Cell numbers required for high quality library
164 generation will vary with the transcription activity of each cell type, with fast-dividing cultured
165 cells typically showing the greatest activity.
166
167 We performed the new qPRO-seq protocol alongside the original protocol in two biological
168 replicates of 10^6 K562 cells (Fig. 3). We observe that the C1 beads decrease the bias against
169 long RNAs seen with M280 beads, resulting in a relatively higher capture rate of gene body
170 reads which results in lower overall pause indices (pause index is pause region signal divided
171 by length-normalized gene body signal, so increased gene-body capture rate and unchanged
172 pause region capture rate decreases pause index; Fig. 3G). In aggregate profiles, we observe
173 no detectable difference in promoter or enhancer profiles across protocols (Fig. 3E–F). Though
174 the overall number of unique reads obtained are lower in libraries made with 0.25 x 10^6 cells as
175 is expected with scarce input material, we still observe transcription at many enhancers (Fig.
176 3F). We find that both assays can reproducibly quantify both gene body and promoter-proximal
177 polymerases (Fig. 3H–I) and discover similar sets of regulatory elements using dREG (Wang et
178 al., 2019). The promoter region is more inherently variable, but using principal component
179 analysis, we were able to determine that when compared to published, deeply sequenced PRO-
180 seq data in K562 cells (Core et al., 2014), batch effect and/or sequencing depth accounted for
181 >99% of variance in both the gene body and promoter region, while choice of protocol (PRO-
182 seq or qPRO-seq) accounted for less than 0.3% of variance (Fig. 3J). This improved and
183 optimized protocol expands the utility and accessibly of PRO-seq without sacrificing precision or
184 data quality.
185

186 **Data Availability**

187 K562 PRO-seq and qPRO-seq raw sequencing data, processed bigWig files and dREG peaks
188 files have been deposited in GEO (GSE150625).
189

190 **Code Availability**

191 Code used to analyze data and generate figures in this manuscript is available at
192 https://github.com/JAJ256/qPRO. The pipeline used for PRO-seq alignment and processing is
193 available at https://github.com/JAJ256/PROseq_alignment.sh. “Browser shots” were generated
194 using code found here: https://github.com/JAJ256/browser_plot.R.
195

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196 **Author Contributions**

197 J.J. performed PRO-seq and qPRO-seq experiments and analyzed data. J.J., L.J.C., L.M.W,
198 and L.A.W wrote and revised the manuscript. L.A.W., L.M.W., G.J.V., and A.D. optimized on-
199 bead steps biochemical steps. All authors reviewed and approved the final manuscript. E.J.R.
200 contributed the qPCR protocol. E.M.W. performed experiments to optimize the protocol for low-
201 input experiments. All authors contributed experimentally or conceptually to the development of
202 this protocol.

203

204 **Protocol Availability**

205 The protocol presented in this manuscript is also available at
206 https://dx.doi.org/10.17504/protocols.io.57dg9i6 and will be updated as further improvements
207 are made.

208

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218 ## 2. Materials

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

221 ### 2.1 Chemicals and Reagents

222 1. Diethyl pyrocarbonate (DEPC; Sigma-Aldrich, cat. no. D5758)
223 ! **CAUTION:** DEPC is toxic and harmful
224 2. DEPC treated ddH_2O (0.1% v/v)
225 3. 4 M KCl (see Note 1)
226 4. 5 M NaCl (see Note 1)
227 5. 1M $MgCl_2$ (see Note 1)
228 6. 10% (v/v) Triton X-100 (see Note 2)
229 7. 2% (v/v) Sarkosyl (Sigma-Aldrich, cat. no. L5125) (see Note 2)
230 8. 2% (v/v) Tween-20 (see Note 2)
231 9. 10 % Igepal® CA-630 (Millipore Sigma cat. no. I8896) (see Note 2)
232 10. 0.1 M DTT (see Note 2)
233 11. 1 M Sucrose (see Note 2)
234 12. 0.5 M EDTA, pH 8.0 (see Note 2)
235 13. 0.1 M EGTA, pH 8.0 (see Note 2)
236 14. Tris-Cl, pH 6.8 (See Note 2)
237 15. Tris-Cl, pH 7.4 (See Note 2)
238 16. Tris-Cl, pH 8.0 (See Note 2)
239 17. Glycerol
240 18. 1 N NaOH
241 19. 100% Ethanol
242 20. 75% Ethanol
243 21. Biotin-11-CTP 10 mM (PerkinElmer, cat. no. NEL542001EA)
244 22. Biotin-11-UTP 10 mM (PerkinElmer, cat. no. NEL543001EA)
245 23. Biotin-11-GTP 10 mM (PerkinElmer, cat. no. NEL545001EA)
246 24. Biotin-11-ATP 10 mM (PerkinElmer, cat. no. NEL544001EA)
247 25. GTP, 100 mM (Roche, cat. no. 11277057001)
248 26. ATP, 100 mM (Roche, cat. no. 11277057001)
249 27. Trypan Blue
250 28. Dynabeads™ MyOne™ Streptavidin C1 Beads (Invitrogen, cat. no. 65002)
251 29. TRIzol™ Reagent (Invitrogen, cat. no. 15596018)
252 30. TRIzol™ LS Reagent (Invitrogen, cat. no. 10296028) (optional, see Note 3)
253 31. Total RNA Purification Kit (Norgen, cat. no. 37500) (optional, see Note 3)
254 32. Chloroform

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255 33. GlycoBlue™ (Invitrogen, cat. no. AM9515)
256 34. SUPERase-In™ RNase Inhibitor (20 U/μL) (Invitrogen, cat. no. AM2694)
257 35. Pierce™ Protease Inhibitor Tablets (Thermo Scientific cat. no. A32963)
258 36. T4 RNA Ligase (10 U/μL), supplied with 10X T4 RNA ligase buffer, 10 mM
259 ATP and 50% (w/v) PEG8000 (NEB cat. no. M0204S)
260 37. RNA 5' pyrophosphohydrolase (RppH; 5 U/μL) (NEB, cat. no. M0356S)
261 38. 10X ThermoPol Reaction Buffer (NEB, cat. no. B9004S)
262 39. T4 polynucleotide kinase (PNK; 10 U/μL), supplied with 10X PNK buffer
263 (NEB, cat. no. M0201)
264 40. Maxima H Minus Reverse Transcriptase (200 U/μL) (Thermo Scientific, cat.
265 no. EP0751)
266 41. dNTP mix, 12.5mM each (Roche, cat. no. 03622614001)
267 42. Q5® High-Fidelity DNA Polymerase, supplied with 5X Q5 Reaction Buffer
268 and 5X High GC-Enhancer (NEB cat. no. M0491S)
269 43. Micro Bio-Spin™ RNase free P-30 Gel Columns (Bio-Rad, cat. no.
270 7326250) (optional, see Note 3)
271 44. RNA and DNA oligos (IDT DNA) (see Table 1 and Notes 4–6)
272 45. Agencourt AMPure XP SPRI beads (Beckman-Coulter, cat. no. A63880)
273 46. SYBR Gold stain (Invitrogen, cat. no. S11494)
274 47. Costar® Spin-X® 0.22 μm Centrifuge Tube Filters (Corning, cat. no. 1860)
275 48. SsoAdvanced Universal SYBR Green Supermix (BioRad, cat. no. 1725270)
276
277 **2.2 Buffers**

278 1. Permeabilization Buffer: 10 mM Tris-Cl [pH 8.0] ,10 mM KCl, 250 mM
279 Sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% (v/v) Igepal, 0.5 mM DTT, 0.05%
280 (v/v) Tween-20, and 10% (v/v) Glycerol in DEPC H_2O. Add 1 Pierce
281 protease inhibitor tablet and 10 μl SUPERase-In RNase inhibitor per 50 mL
282 (see Note 7)
283 2. Cell Wash buffer: 10 mM Tris-Cl [pH 8.0], 10 mM KCl, 250 mM sucrose,
284 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, and 10% (v/v) Glycerol in DEPC
285 H_2O. Add 1 Pierce protease Inhibitor tablet and 10 μl SUPERase-In RNase
286 inhibitor per 50 mL (see Note 7)
287 3. Freeze buffer: 50 mM Tris-Cl [pH 8.0], 40% (v/v) glycerol, 5 mM $MgCl_2$,
288 1.1 mM EDTA, and 0.5 mM DTT in DEPC H_2O. Add 10 μl SUPERase-In
289 RNase inhibitor per 50 mL (see Note 7)
290 4. Bead Preparation Buffer: 0.1 N NaOH and 50 mM NaCl in DEPC H_2O.
291 5. Bead Binding Buffer: 10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 0.1% (v/v)
292 Triton X-100, 1 mM EDTA in DEPC H_2O. Add 2 μl SUPERase-In RNase

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293 Inhibitor per 10 mL (see Note 7)

294 6. High Salt Wash buffer: 50 mM Tris-HCl [pH 7.4], 2 M NaCl, 0.5% (v/v) Triton
295 X-100, and 1 mM EDTA in DEPC H_2O. Add 2 μl SUPERase-In RNase
296 Inhibitor per 10 mL (see Note 7)

297 7. Low Salt Wash Buffer: 5 mM Tris-HCl [pH 7.4], 0.1% (v/v) Triton X-100, and
298 1 mM EDTA in DEPC H_2O. Add 2 μl SUPERase-In RNase Inhibitor per 10
299 mL (see Note 7)

300 8. 2X Run-On Master Mix (2XROMM): 10 mM Tris-Cl [pH 8.0], 5 mM $MgCl_2$, 1
301 mM DTT, 300 mM KCl, 40 μM Biotin-11-CTP, 40 μM Biotin-11-UTP, 40 μM
302 Biotin-11-ATP, 40 μM Biotin-11-GTP, 1% (w/v) Sarkosyl in DEPC H_2O. Add 1
303 μL SUPERase-In RNase Inhibitor per reaction (see Note 8).

304

305 **2.3 Consumables**

306 1. Low-bind, nuclease-free microcentrifuge tubes (0.5 and 1.5 mL)

307 2. Low-bind, nuclease-free, filtered pipette tips

308 3. Wide bore, low-bind, nuclease-free, filtered P1000 and P200 tips (or cut
309 standard bore ~1cm from tip)

310

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311 # 3. Methods

312 ## 3.1 Cell Permeabilization

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

314 1. Prepare permeabilization buffer, wash buffer, and freeze buffer and place on
315 ice (see Note 7).
316 2. Proceed using one of the following options:
317
318 Option 3.1.2.1: Adherent cells (volumes are for 10 cm plates):
319 1. Wash cells with 10 mL ice cold PBS.
320 2. Repeat the PBS wash step for a total of two washes.
321 3. Add 5 mL ice cold permeabilization buffer, scrape cells, and transfer
322 to a conical tube.
323 4. Rinse plate with 5 mL permeabilization buffer and pool cells in conical
324 tube (V_f = 10 mL).
325
326 Option 3.1.2.2: Suspension cells:
327 1. Transfer cells into conical tube and spin down at 700–1000 x g for 4
328 min at 4°C (see Notes 9–10).
329 2. Wash with 10 mL ice cold PBS (see Notes 10–11).
330 3. Repeat the PBS wash for a total of two washes.
331 4. Resuspend in 10 mL cold permeabilization buffer (see Note 11).
332
333 3. Continue here from step 3.1.2.1.4 or 3.1.2.2.4.
334 4. Incubate on ice for 5 min.
335 5. Check for permeabilization with Trypan blue. Greater than 98%
336 permeabilization is ideal (see Note 12).
337 6. Spin down at 700–1000 x g for 4 min at 4°C (see Notes 9–10).
338 7. Wash with 10 mL ice cold cell wash buffer (see Notes 10–11).
339 8. Repeat the cell wash buffer wash for a total of two washes.
340 9. Decant wash buffer, and then carefully pipette off remaining buffer and
341 discard without disturbing the cell pellet.
342 10. Using wide-bore tips, resuspend in 250 μL cold freeze buffer and transfer to
343 a 1.5 mL tube.
344 11. Rinse the conical tube with an additional 250 μL freeze buffer and pool (V_f =
345 500 μL).
346 12. Count cells and add permeabilized spike-in cells if desired (see Notes 13–
347 14).

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13. Spin down at 1000 x g for 5 min at 4°C (see Note 15).
14. Resuspend the desired number of cells for each run-on reaction in 52 μL freeze buffer (see Note 16).
15. Continue to the run-on or snap freeze 52 μL aliquots in LN_2 and store at -80°C (see Note 17).

3.2 Preparation

1. Pre-chill a microcentrifuge to 4°C.
2. Set a heat block with water in the wells to 37°C and another to 65°C and allow temperature to equilibrate.
3. Prepare bead preparation buffer, high salt wash buffer, low salt wash buffer, and binding buffer (see Note 7).
4. 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 (see Notes 18–19).
5. Wash beads twice with 1 mL binding buffer (see Note 19–20).
6. Resuspend the beads in 25 µL binding buffer per sample. Place beads on ice or at 4°C until needed.

3.3 Run-On Reaction

1. Prepare 2XROMM equilibrate at 37°C (30°C for Drosophila) (see Note 8 and 21).
2. Using a wide bore tip, add 50 μL of permeabilized cells to new 1.5 mL tube.
3. Pipette 50 μL of preheated 2XROMM into each reaction tube (already containing permeabilized cells). *Gently and thoroughly pipette the mixture 15 times*. It is extremely important to thoroughly mix the reaction so that nucleotides diffuse into highly viscous chromatin!
4. Incubate in a thermomixer at 37°C (30°C for Drosophila) at 750 RPM for 5 min. Have RL buffer from Norgen kit or TRIzol LS ready for use.
5. Proceed to step 3.4.1.1 or 3.4.1.2 depending on choice of RNA extraction method immediately after the 5 min reaction is complete (take the sample off the heat block and immediately add buffer RL or TRIzol LS).

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381 Use the table below to coordinate timing of run-on reaction and addition of TRIzol
382 or Buffer RL. Start the timer (counting up) when you add the first sample:

Time	Step
00:00	Mix permeabilized cells with 2XROMM for Sample 1
01:00	Mix permeabilized cells with 2XROMM for Sample 2
02:00	Mix permeabilized cells with 2XROMM for Sample 3
03:00	Mix permeabilized cells with 2XROMM for Sample 4
04:00	Mix permeabilized cells with 2XROMM for Sample 5
05:00	Add buffer RL or TRIzol LS to Sample 1, vortex briefly
06:00	Add Buffer RL or TRIzol LS to Sample 2, vortex briefly
07:00	Add Buffer RL or TRIzol LS to Sample 3, vortex briefly
08:00	Add Buffer RL or TRIzol LS to Sample 4, vortex briefly
09:00	Add Buffer RL or TRIzol LS to Sample 5, vortex briefly

383

384 **3.4 Total RNA Extraction and Base Hydrolysis**

385 1. Proceed from step 3.3.5 to one of the following options:

386

387 <u>Option 3.4.1.1: NORGEN RNA Extraction (see Note 3):</u>

388 1. Add 350 μL RL buffer and vortex.
389 2. Add 240 μL 100% ethanol and vortex.
390 3. Apply solution to Norgen RNA extraction column.
391 4. Spin at 3,500 x g for 1 min at 25°C.
392 5. Add 400 μL wash solution A (ensure ethanol has been added).
393 6. Spin at 14,000 x g for 1 min at 25°C.
394 7. Discard flow through.
395 8. Repeat wash (steps 6 & 7) for a total of two washes.
396 9. Spin at 14,000 x g for 2 min to dry column.
397 10. Add 50 μL DEPC H_2O and vortex.
398 11. Elute by spinning at 200 x g for 2 min at 25°C and then at 14,000 x g
399 for 1 min at 25°C.
400 12. Elute again with 50 μL H_2O and pool eluates (V_f = 100 μL).
401 13. Denature at 65°C for 30 sec and then snap cool on ice.
402 14. Add 25 μL ice cold 1 N NaOH and incubate 10 min on ice.
403 15. Add 125 μL cold 1 M Tris-Cl pH 6.8, mix by pipetting.
404 16. Add 5 μL 5 M NaCl and 1 μL GlycoBlue and mix.
405 17. Add 625 μL 100% Ethanol and vortex (see Note 22).
406 18. Centrifuge the samples at >20,000 x g for 20 minutes at 4°C (see

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407 Note 23).
408 19. Carefully pipette supernatant off and discard (see Note 24).
409 20. Add 750 μL 70% ethanol.
410 21. Mix by gentle inversion and spin down briefly.
411 22. Carefully pipette supernatant off and discard (see Note 24).
412 23. Airdry the RNA pellet (see Note 25).
413 24. Resuspend in 6 μL DEPC H_2O.
414
415 Option 3.4.1.2: Trizol LS RNA Extraction (See Note 3):
416 1. Add 250 μL TRIzol LS with a wide bore P1000 tip, pipette vigorously
417 but carefully >10X until all white globs of nucleoproteins are
418 homogenized.
419 2. Pipette mix again with a standard bore P1000 tip. Samples should be
420 completely homogenous.
421 3. Vortex vigorously for at least 15 seconds.
422 4. Incubate samples on ice until all run-on reactions are complete.
423 5. Add 65 μL chloroform (see Note 26).
424 6. Vortex the samples at max speed for 15 sec, then incubate on ice for
425 3 min.
426 7. Centrifuge the samples at >20,000 x g for 8 min at 4°C.
427 8. Transfer the ~200 μL aqueous phase into a new tube (see Note 27).
428 9. Add 1 μL of GlycoBlue and mix.
429 10. Add 2.5X volumes (~500 μL) 100% ethanol and vortex.
430 11. Centrifuge at >20,000 x g for 20 min at 4°C (see Note 23).
431 12. Carefully pipette supernatant off and discard (see Note 24).
432 13. Add 750 μL 70% ethanol.
433 14. Mix by gentle inversion and quickly spin down.
434 15. Carefully pipette supernatant off and discard (see Note 24).
435 16. Airdry the RNA pellet (see Note 25).
436 17. Resuspend in 30 μL DEPC H_2O.
437 18. Briefly denature at 65°C for 30 sec and then snap cool on ice.
438 19. Add 7.5 μL ice cold 1 N NaOH and incubate on ice for 10 min.
439 20. Add 37.5 μL 1 M TrisCl pH 6.8, mix by pipetting.
440 21. Pass through a calibrated Bio-Rad RNase free P-30 column (follow
441 manufacturer's instructions).
442 22. Bring volume to 200 μL with DEPC H_2O (add ~ 125 μL).
443 23. Add 1 μL Glycoblue and 8 μL 5 M NaCl and vortex.
444 24. Add 500 μL 100% ethanol and vortex (see Note 22).

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445 25. Centrifuge at >20,000 x g for 20 minutes at 4°C (see Note 23).
446 26. Carefully pipette supernatant off and discard (see Note 24).
447 27. Add 750 μL 70% ethanol.
448 28. Mix by gentle inversion and quickly spin down.
449 29. Carefully pipette supernatant off and discard (see Note 24).
450 30. Airdry the RNA pellet (see Note 25).
451 31. Resuspend in 6 μL DEPC H_2O.
452

453 **3.5 3' RNA Adaptor Ligation**

454 1. Continue here from step 3.4.1.1.24 or 3.4.1.2.31:
455 2. Add 1 μL 10 μM VRA3 (see Note 28).
456 3. Denature at 65°C for 30 sec and snap cool on ice.
457 4. Prepare ligation mix in the following order (see Note 29):
458

Reagent	Volume
10X T4 RNA ligase buffer	2 μL
ATP (10 mM)	2 μL
SUPERase-In RNase Inhibitor	1 μL (see Note 30)
50% PEG8000	6 μL
T4 RNA Ligase I (NEB)	2 μL

459
460 5. Add 13 μL and mix by pipetting 10–15X (V_f = 20 μL).
461 6. Incubate at 25°C for 1 h.
462

463 **3.6 Streptavidin Bead Binding**

464 1. Add 55 μL binding buffer to each sample (V_f = 75 μL).
465 2. Add 25 μL pre-washed beads to each sample (V_f = 100 μL).
466 3. Incubate for 20 min at 25°C with end to end rotation.
467 4. Wash once with 500 μL High Salt Wash buffer with tube swap (see Notes
468 19, 31–32).
469 5. Wash once with 500 μL Low Salt Wash buffer (see Notes 19, 31, 33).
470

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471 **3.7 On-Bead 5' Hydroxyl Repair**

472 1. Resuspend beads in 19μL PNK mix (V_f = 20 μL; see Note 34):

473

Reagent	Volume
DEPC H_2O	13 μL
10X PNK buffer	2 μL
10 mM ATP	2 μL
T4 PNK (NEB)	1 μL
SUPERase-IN RNase Inhibitor	1 μL (see Note 30)

474

475 2. Incubate at 37°C for 30 min (see note 35).

476

477 **3.8 On-Bead 5' Decapping**

478 1. Place the tubes on a magnet stand and remove supernatant (see Notes 19–
479 20, 33).

480 2. Resuspend the beads in 19 μL RppH mix (V_f = 20 μL; see Notes 34, 36):

481

Reagent	Volume
DEPC H_2O	15 μL
10X ThermoPol Buffer	2 μL
RppH	1 μL
SUPERase-In RNase Inhibitor	1 μL

482

483 3. Incubate at 37°C for 1 h (see note 35).

484

485 **3.9 On-Bead 5' RNA Adaptor Ligation**

486 1. Place the tubes on a magnet stand and remove supernatant (see Notes 19–
487 20, 33).

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

489

Reagent	Volume
DEPC H_2O	6 μL
REV5 (10 μM) (see Note 28)	1 μL

490

491 3. Denature at 65°C for 30 sec, then snap cool on ice.

492 4. Prepare ligation mix in the following order (see Note 29):

493

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Reagent	Volume
10x T4 RNA ligase buffer	2 μL
ATP (10 mM)	2 μL
SUPERase-In RNase Inhibitor	1 μL (see Note 30)
50% PEG8000	6 μL
T4 RNA ligase I	1 μL

494
495 5. Add 12 μL to each tube (V_f = 20 μL).
496 6. Incubate at 25°C for 1 h (see Note 35).
497

498 **3.10 TRIzol Elution of RNA**

499 1. Wash once with 500 μL High Salt Wash buffer with tube swap (see Notes
500 19, 31–32).
501 2. Wash once with 500 μL Low Salt Wash buffer (see Notes 19, 31, 33).
502 3. Resuspend beads in 300 μL TRIzol.
503 4. Vortex at max speed for >20 sec, then incubate on ice for 3 min.
504 5. Add 60 μL chloroform (see Note 26).
505 6. Vortex at max speed for 15 sec, then incubate on ice for 3 min.
506 7. Centrifuge at >20,000 x g for 8 min at 4°C.
507 8. Transfer the aqueous phase (~180 μL) to a new tube (see Note 27).
508 9. Add 1 μL GlycoBlue and mix.
509 10. Add 2.5X volumes (~450 μL) 100% ethanol and vortex.
510 11. Centrifuge the samples at >20,000 x g for 20 min at 4°C (see Note 23).
511 12. Carefully pipette supernatant off and discard (see Note 24).
512 13. Add 750 μL 70% ethanol.
513 14. Mix by gentle inversion and quickly spin down.
514 15. Carefully pipette supernatant off and discard (see Note 24).
515 16. Airdry the RNA pellet (see Note 25).
516

517 **3.11 Off-Bead Reverse Transcription**

518 1. Resuspend RNA pellet in 13.5 μL RT resuspension mix (see Note 37):
519

Reagent	Volume
DEPC H_2O	8.7 μL
RP1 (10 μM)	4
dNTP mix (12.5 mM each)	0.8μL

520
521 2. Denature at 65°C for 5 min and snap cool on ice.

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3. Prepare RT master mix:

Reagent	Volume
5X RT Buffer	4 μL
100 mM DTT	1 μL
SUPERase-In RNase Inhibitor	0.5 μL (see Note 30)
Maxima H Minus RT enzyme	1 μL

4. Add 6.5 μL to each sample (V_f = 20 μL).
5. Cycle as follows: 50°C for 30 min, 65°C for 15 min, 85°C for 5 min, then hold at 4°C.
6. Immediately proceed to PreCR, test amplification, or full-scale amplification. Samples can be stored overnight at -20°C (see Notes 38–39).

3.12 PreCR (Optional, see Note 38)

1. Add 2.5 μL RPI-n indexed primer (10 μM) to each sample. Use different barcodes for samples that will be pooled and sequenced together.
2. Prepare the PreCR master mix:

Reagent	Volume
ddH_2O	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 (NEB)	1 μL

3. Add 77.5 μL of the PreCR mix to each sample for final volume 100 μL (see Note 40).
4. Amplify libraries for 5 cycles on thermal cycler using the following settings:
   a. 95°C for 2 min
   b. 95°C for 30 sec
   c. 56°C for 30 sec
   d. 72°C for 30 sec
   e. Go to step 2 for 4 more times
   f. 72°C for 5 min
   g. Hold at 4°C.

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

3.13 Test Amplification (Gel) (Optional, see Note 39)

1. Make the first dilution:
   a. 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 (see Note 41).
   b. 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 (see Note 41).
2. Make 4-fold serial dilutions by adding 2 µL of each dilution to 6 µL ddH_2O for the next dilution.
3. Remove and discard 2 µL from the final dilution (all dilutions should now be 6 µL).
4. 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

5. Make test PCR mix:

Reagent	Volume
H_2O	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 (NEB)	0.2 µL

6. Add 14µL PCR mix to the 6 µL diluted test samples (V_f = 20 µL).
7. Amplify reactions for the desired amount of cycles using following settings:
   ! **CRITICAL** Remember to account for PreCR. Subtract 5 cycles from your total target test amplification cycles.
   a. 95°C for 2 min

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573 b. 95°C for 30 sec
574 c. 65°C for 30 sec (see Note 43)
575 d. 72°C for 30 sec
576 e. Go to step 2 for the desired number of cycles
577 f. 72°C for 5 min
578 g. Hold at 4°C
579 8. Mix with gel loading dye to 1X and run 10 μL on a 2.2% Agarose gel or run 2
580 μL on a native 8% polyacrylamide gel and stain with SYBR Gold.
581 9. Use the test amplification gel to determine the appropriate number of cycles
582 for full-scale amplification (see Note 44).
583
584 **3.14 Test Amplification (qPCR) (Optional, see Note 39)**
585 1. Add 1.54 μL of the 20 μL RT reaction to 0.46 μL ddH_2O (V_f = 2 μL).
586 2. Make the qPCR master mix:
587

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
H_2O	2.5 μL

588
589 3. Add 8 μL of the qPCR master mix to 2 μL diluted RT reaction (V_f = 10 μL).
590 4. Quickly spin plate to collect liquid.
591 5. Amplify in a real-time PCR system using the following conditions:
592 a. Amplification
593 i. 98 °C for 2 min
594 ii. 98 °C for 15 sec
595 iii. 60 °C for 60 sec
596 iv. Go to step 2 for 39 additional cycles
597 b. Melting Curve
598 i. 95 °C for 15 sec
599 ii. 60 °C for 1 min
600 iii. 96 °C for 15 sec
601 iv. 60 °C for 16 sec
602 6. Calculate the number of full-scale amplification cycles as the cycle number
603 where Rn reaches 0.25 × Rn_{max}.
604
605

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606 **3.14 Full-Scale Amplification**

607 1. If PreCR and Test Amplification were skipped:
608 a. Add 2.5 μL of an RPI-n indexed primer (10μM) to each 20 μL RT
609 reaction. Use different barcodes for samples that will be pooled and
610 sequenced on a single lane.
611 b. Prepare the PCR master mix in step 2 of section 3.12 (PreCR).
612 c. Add 77.5 μL PCR master mix to each sample for final volume 100 μL
613 (see Note 40).
614 d. Run the desired number of cycles (see below)
615 2. If PreCR was skipped but Test Amplification was performed:
616 a. Add 2.5 μL of an RPI-n indexed primer (10μM) to the remaining
617 18.5 μL RT reaction. Use different barcodes for samples that will be
618 pooled and sequenced on a single lane.
619 b. Prepare the PCR master mix in step 2 of section 3.12 (PreCR) but
620 use 35 μL ddH_2O instead of 33.5 μL.
621 c. Add 79 μL PCR master mix to each sample for final volume 100 μL
622 (see Note 40).
623 d. Run the desired number of cycles (see below).
624 3. If PreCR and Test Amplification were performed:
625 a. Prepare the following spike-in PCR mix:
626

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Reagent	Volume
ddH_2O	3.7 µL
5x Q5 buffer	1.5 µL
5x Q5 enhancer	1.5 µL
dNTP mix (12.5mM each)	0.5 µL
Q5 polymerase (NEB)	0.5 µL

627
628 b. Add 7.7 µL PCR spike-in mix to each sample for final volume 100 µL
629 (see Note 40).
630 c. Run the desired number of cycles.
631 4. Amplify for the desired number of cycles:
632 ! **CRITICAL:** Remember to account for PreCR. Subtract 5 cycles from
633 your total target full-scale amplification cycles.
634 a. 95°C for 2 min
635 b. 95°C for 30 sec
636 c. 65°C for 30 sec (see Note 43)
637 d. 72°C for 30 sec
638 e. Go to step 2 for the desired number of cycles
639 f. 72°C for 5 min
640 g. Hold at 4°C
641 5. Allow PCR reactions to reach room temperature.
642 6. Add 180 µL SPRI beads (see Note 45) at room temperature and
643 immediately mix by pipetting > 15X.
644 7. Incubate at room temperature for 5 min.
645 8. Place on a magnet stand and remove the supernatant.
646 9. Wash the beads twice with 70% Ethanol without resuspending.
647 **! CRITICAL:** Do not disturb the beads or library recovery will be greatly
648 reduced.
649 10. Airdry the beads for 5 min. Do not over dry the beads.
650 11. Resuspend beads in 22 µL 10 mM Tris-Cl, pH 8.0 (no EDTA).
651 12. Incubate at room temperature for 5 min.
652 13. Place the beads on a magnet stand and transfer 20 µL to a new tube.
653 14. Quantify the library using the Qubit dsDNA-HS assay and run on a
654 Bioanalyzer.
655
656 **3.15 PAGE purification (optional, see Note 46)**
657 1. Add Orange G loading dye to 1X to the entire library volume.

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658 2. Run the samples on a native 8% polyacrylamide gel.
659 3. Stain with SYBR Gold.
660 4. Cut a gel slice from immediately above the adapter dimer to ~650 bp (see
661 Note 44).
662 5. Place the gel slice in a 0.5 mL microfuge tube.
663 6. Make a hole in the bottom of the tube with an 18G needle.
664 7. Nest the 0.5 mL tube in a 1.5 mL tube and spin at 5000 x g for 1 min.
665 8. If gel remains in the 0.5 mL tube, repeat step 7 and pool shredded gel
666 fractions by suspending each in 250 μL soaking buffer using a wide-bore
667 P1000 tip.
668 9. Soak the gel pieces in 0.5 mL soaking buffer (TE + 150mM NaCl + 0.1%
669 Tween-20) overnight with agitation at 37°C.
670 10. Spin the tube at 5000 x g for 1 min.
671 11. Pipette as much of the soaking buffer as possible without transferring gel
672 pieces into a new tube.
673 12. Add an additional 0.5 mL soaking buffer and incubate 4 h at 37°C with
674 agitation.
675 13. Spin the tube at 5000 x g for 1 min.
676 14. Pipette as much of the soaking buffer as possible without transferring gel
677 pieces into the tube with the previous eluate.
678 15. Pass the remaining gel solution through a Costar Spin-X column using a cut
679 P1000 tip and pool with the previous eluate (V_f = 1 mL)
680 16. Reduce the volume by half (V_f = 0.5 mL) using vacuum dryer at 37°C.
681 17. Add 1uL GlycoBlue.
682 18. Add 2.5X volume (1.25 mL) 100% ethanol and vortex.
683 19. Centrifuge at >20,000 x g for 20 minutes at 4°C (see Note 23).
684 20. Carefully pipette off the supernatant and discard (see Note 24).
685 21. Add 750μL of 75% ethanol.
686 22. Mix by gentle inversion and quickly spin down.
687 23. Carefully pipette off the supernatant and discard (see Note 24).
688 24. Air-dry the RNA pellet (see Note 25).
689 25. Resuspend the pellet in the desired volume of 10 mM Tris-Cl, pH 8.0 (no
690 EDTA!).
691

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692 ## 4. Notes

693 1. All salt solutions should be prepared in ddH_2O. Then add 0.1% (v/v) DEPC, stir
694 overnight, and autoclave. Tris buffers instead need to be carefully prepared with
695 DEPC-treated ddH_2O.
696 2. All other solutions (detergents, DTT, sucrose, EDTA/EGTA, and Tris buffers) should
697 be prepared in DEPC ddH_2O in RNase free containers and filter sterilized.
698 Glassware can be made RNase by filling with water, adding 0.1% (v/v) DEPC,
699 incubating with agitation overnight, and autoclaving. Alternatively, glassware can
700 be baked at 300 °C for 4 hours.
701 3. TRIzol LS or the Norgen Total RNA Purification Kit can be used to extract total
702 RNA from the run-on reaction. Both options produce identical results. The Norgen
703 kit is faster and less technically challenging to use, but more expensive. If TRIzol
704 LS is used, Micro Bio-Spin™ RNase free P-30 Gel Columns are also needed to
705 remove unincorporated biotin-NTPs as the biotin concentration will otherwise
706 overwhelm the binding capacity of the streptavidin beads.
707 4. REV3 and REV5 sequences are reverse complements of the standard RA3/RA5
708 adapter design used in the Illumina TruSeq small RNA library prep kit with added
709 6XN UMIs and a final ligation-optimal fixed base. The oligos are blocked from
710 additional ligation by an inverted dT/ddT group. This design results in sequencing
711 of the 3' end of the nascent RNA at the beginning of read 1 using standard Illumina
712 sequencing primers. Standard RA3/RA5 adapters (custom synthesized or from
713 compatible library prep kits) can be substituted, but in this case paired end
714 sequencing is necessary as the 3' end of the nascent RNA will be sequenced at
715 the beginning of read 2.
716 5. Index sequences in RPI-n primers are standard 6 nt Illumina TruSeq indices. If
717 multiplexing of more than 12 libraries is desired, additional indexed primers can be
718 designed using additional TruSeq 6 nt index sequences. The index sequences
719 must be inserted in the primer reverse complemented because the RPI-n primers
720 are the reverse primers in the library PCR reaction.
721 6. If desired, specialized REV3 oligos can be designed to facilitate additional in-line
722 barcoding to allow for the pooling of multiple samples after the 3' ligation, which
723 simplifies downstream handling. We have had success with replacing the UMI
724 sequence (rNrNrNrNNN) with known barcode sequences followed by the final fixed
725 rU. These barcodes are then the first 6 sequenced nucleotides of Read 1 and can
726 be used to computationally demultiplex samples.
727 7. The permeabilization buffer, cell wash buffer, freeze buffer, and bead
728 washing/binding buffers can be made and filter-sterilized in advance without the
729 DTT, SUPERase-In™ RNase Inhibitor, and Pierce™ protease inhibitor tablets.

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730 DTT, SUPERase-In™ RNase Inhibitor, and protease inhibitor tablets can be added
731 when buffers are needed. Store buffers at 4°C. Use DEPC treated glassware or
732 RNase free plasticware.
733 8. The run-on reaction uses 4 biotin-NTPs. However, ATP and GTP can be
734 substituted at equal concentration for Biotin-11-ATP and Biotin-11-GTP to reduce
735 cost. Biotin-11-ATP and biotin-11-GTP are 10X as expensive as biotin-11-CTP and
736 biotin-11-UTP. With two biotin-NTPs blocking elongation, each polymerase can be
737 expected to extend ~5 nt or less which we find gives sufficient resolution for the
738 vast majority of applications. For low cell number experiments, increase the
739 concentration of the biotin-NTPs to 500 μM. Biotin-NTP incorporation efficiency is
740 ~60% with the concentration in the 2XROMM as written, which is sufficient for
741 experiments using 10^6 cells or greater, but increasing the concentration improves
742 incorporation to ~77% (data not shown).
743 9. Use a centrifuge with a swinging bucket rotor for all centrifuge steps during cell
744 permeabilization. Using a fixed angle rotor will shear cells, releasing a smear of
745 white chromatin.
746 10. Centrifuge speed is cell size dependent. We typically centrifuge HeLa at 800 x g
747 and Drosophila at 1,000 x g.
748 11. When resuspending cells during permeabilization after centrifugation steps, first
749 gently resuspend the cell pellet with 1 mL solution with a wide-bore P1000 tip.
750 Then add the remaining volume (usually 9 mL) and mix by gentle inversion.
751 12. If your cell type is not permeabilized under these conditions, add Triton X-100 to
752 0.1-0.2%.
753 13. When processing multiple samples, if counting will cause the cells to sit on ice for
754 greater than 10 min, reserve 10 μL for counting, aliquot cells in 100 μL aliquots,
755 and snap freeze. Count the cells and then adjust the concentration with freeze
756 buffer after thawing and prior to the run-on.
757 14. In order to robustly normalize between conditions where a dramatic change in
758 global transcription levels are expected, we add a fixed number of cells of a
759 different species to a fixed number of experimental cells at the permeabilization
760 step. Reads can be mapped to a combined genome, and the number of spike-in
761 mapped reads can then be used as a scaling factor. These cells should be
762 permeabilized prior to the experiment, aliquoted, and added to 1-2% by cell
763 number after permeabilization and counting, either just prior to freezing or just prior
764 to the run-on reaction. We frequently use Drosophila S2 cells to normalize human
765 cell experiments and vice versa.

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15. Eppendorf 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).
16. 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.
17. Permeabilized cells are stable indefinitely at -80°C (Chu et al., 2018).
18. 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.
19. 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.
20. Always quickly spin samples down using a picofuge to remove liquid from tube caps.
21. 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.
22. If the protocol needs to be performed over two days, the ethanol precipitation in 3.4 is the safest overnight stopping point. After step 17 of 3.4.1 or step 24 of section 3.4.2, samples can be stored at -80 °C. The protocol can also be stopped after the ethanol precipitation following TRIzol extractions, but samples must be stored for at most one night at 4 °C because at -20 °C precipitation of guanidinium salts can occur and interfere with enzymatic reactions. Alternatively, the RNA pellet can be stored at -80 °C after the precipitation and removal of the supernatant.
23. 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.
24. 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.

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804 25. Air dry the RNA pellet by leaving tubes open in fume hood to prevent
805 contamination. This will take ~3-10 min depending on how much ethanol is left in
806 the tube. Do not to let the RNA pellet dry completely as this will greatly decrease its
807 solubility.
808 26. When pipetting chloroform, always pipette twice because the first draw always
809 leaks.
810 27. When transferring the aqueous phase of TRIzol extractions to a new tube, tilt the
811 tube to a 45° angle and carefully remove only the clear liquid. Avoid contamination
812 by the pink organic phase or white interphase.
813 28. The concentration of RNA adapters in the ligation steps (1 μL 10 μM) is optimal for
814 approximately 10^6 mammalian cells. For lower cell numbers, the adapter
815 concentration must be diluted to limit dimer formation. We dilute linearly with cell
816 concentration relative to this established concentration, i.e. 1 μL 5 μM for 5 x 10^5
817 cells, 1 μL 2.5 μM for 2.5 x 10^5 cells, etc.
818 29. Pipette slowly because 50% PEG8000 is very viscous. Heating 50% PEG8000
819 makes it easier to pipette. Pipette the ligation mix until it is homogenous before
820 use.
821 30. When preparing enzymatic reaction mixtures that contain SUPERase-In RNase
822 Inhibitor, a fixed volume (1 μL) SUPERase-In can be added to the entire master
823 mix regardless of number of reactions to decrease cost. Bring the remainder of the
824 master mix up to the required volume with DEPC H_2O. Murine RNase inhibitor can
825 also be substituted to limit costs for all steps after the run-on. SUPERase-In is
826 recommended prior to the run-on as it inhibits T1 RNase.
827 31. For each washing step gently invert tubes 10–15X, quickly spin down with a
828 picofuge, open caps, and then place on the magnet stand. Wait 1-2 minutes and
829 pipette the supernatant off without disturbing the beads. If there are bubbles in the
830 tube carefully pipette them off first and then remove supernatant. Beware that
831 bubbles may dislodge beads from the side of the tube. After removing the bulk of
832 the liquid, collect remaining liquid with a quick spin in a picofuge, place the tube
833 back on the magnet stand, and carefully remove remaining liquid by pipetting.
834 32. Transferring beads to a new tube after the binding incubation—during the high salt
835 wash step—helps limit adapter dimer formation. After resuspending the beads in
836 High Salt buffer, quickly spin down with a picofuge, resuspend beads by gently
837 pipetting, and carefully transfer to a new tube. Pipette slowly to avoid bead loss!
838 Place this new tube on the magnet stand and proceed with the washing protocol.
839 33. Do not allow streptavidin beads to dry completely, as this can lead to clumping and
840 make full resuspension impossible. When processing multiple samples, remove
841 liquid from the previous wash or enzymatic step from the first sample and

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842 immediately resuspend those beads in the next solution, then repeat this process
843 for additional samples.
844 34. On-bead reaction volumes assume that 1 μL of liquid remains on the beads.
845 35. Mix on-bead reactions by gently flicking the tubes every 10 minutes.
846 36. We have also successfully used Cap-Clip™ Acid Pyrophosphatase (CELLTREAT)
847 instead of RppH. Cap-Clip has lower buffer pH which may alleviate base hydrolysis
848 of RNA that could occur in the pH 8.0 ThermoPol buffer. However, this is not a
849 major concern except for in the most sensitive of applications.
850 37. Reverse transcription can also be performed on-bead, but we find that this
851 significantly reduces library yield while increasing adapter dimer. For this reason, it
852 is not recommended except in cases where material is abundant (10^7 cells) and
853 speed is paramount. To do this, follow steps 1–2 in section 3.12, then follow
854 section 3.13, but resuspend the beads instead of the RNA pellet in RT
855 resuspension mix. After RT, elute cDNA by heating the bead mixture to 95°C,
856 quickly place tubes on a magnet stand, and remove and save supernatant.
857 Resuspend beads in 20 μL ddH_2O and repeat the process for a final volume of 40
858 μL. Proceed with PreCR but use 20 μL less ddH_2O (13.5 μL) in the PreCR mix and
859 use the entire 40 μL eluate instead of the 20 μL RT mix.
860 38. PreCR is optional if full scale amplification will be performed within 2 days. Longer
861 storage of single-stranded cDNA libraries can lead to loss of library material. If you
862 are skipping PreCR, simply store the 20 μL RT reaction at -20°C overnight and
863 perform test amplification the next day.
864 39. Because this protocol uses molecular barcodes (UMIs) which facilitate robust
865 computational PCR deduplication, it is less important to precisely determine the
866 optimal cycle number. We recommend performing test amplification the first time
867 you perform this protocol with a given amount of material from a given cell line to
868 determine the optimal cycle number. For future experiments where the material
869 and cell number are constant, test amplification can be skipped. Adjust the volume
870 of the full-scale PCR to 100 μL total volume (accounting for the fact that the written
871 protocol assumes loss due to test amplification). Test amplification can be
872 performed either by PCR of a dilution curve and PAGE analysis or qPCR.
873 40. Do not attempt to scale down the PreCR or full-scale amplification steps to save
874 PCR reagents. If RT reaction mixture exceeds 20% of the PCR reaction volume,
875 significant inhibition of PCR will occur and lead to dramatically lower final library
876 yield.
877 41. Taking 7.7 μL of the 100 μL PreCR reaction leaves 92.3 μL for full-scale
878 amplification. 25% of material in the first dilution is lost to make the next serial 4-
879 fold dilution (2 of 8 μL). Because $(7.7 * 0.75) / 92.3 \approx 1/16$, this first dilution is

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880 equivalent to the number of test amplification cycles less 4. If starting from the RT
881 reaction, the volume has been adjusted for 5-fold lower starting volume.

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

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

889 44. Desired amplification characteristics include a sufficient amount of product (smear
890 starting ~150 bp), no evidence of overamplification, and ~50% primer exhaustion
891 (Mahat et al., 2016). The adaptor dimer product is 132 bp, and the smear will start
892 15–20 bp above this band. RNA degradation will lead to shorter library products.

893 45. AMPure XP beads will work, as will any commercially available or homemade
894 SPRI bead cleanup reagent based on PEG precipitation. Be sure to allow beads to
895 reach room temperature, or excess primers will also precipitate.

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

902

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986 **Table 1. Oligonucleotides**

Name	Sequence	Purification
REV3	/5Phos/rUrNrNrNrNrNNGATCGTCGGACTGTAGAACTCTGAAC/3InvdT/	RNase-free HPLC
REV5	/5InvddT/CCTTGGCACCCGAGAATTCCANrNrNrNrNrNrC	RNase-free HPLC
RP1	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA	PAGE
RPI-1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-2	CAAGCAGAAGACGGCATACGAGATAGATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-9	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-10	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-11	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE
RPI-12	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA	PAGE

987