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Extended NET-seq Protocol

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We use this protocol and it's working

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Abstract

Here we provide a detailed protocol for NET-seq using *Saccharomyces cerevisiae* with an emphasis on examining the effects of divalent cations on RNA recovery.



Cell growth and harvest

- 1 Streak and grow desired *Saccharomyces cerevisiae* (yeast) strain on a plate.
- 2 Inoculate a starter culture ( 25 mL -  40 mL) and grow overnight to saturation at  30 °C with nutation.  
- 3 Using saturated starter culture, inoculate  1 L of YEPD media and grow culture at  30 °C with nutation until $A_{600} = 0.3$. 
- 4 Meanwhile, set up glass collection apparatus by nesting a glass funnel with a fritted filter into a  1 L Erlenmeyer flask with a side arm.
- 5 Once cells have reached $A_{600} = 0.3$, place a 0.45 μm Cellulose Nitrate Membrane Filter (GE Healthcare – Whatman, #7184-009) on top of the glass funnel and connect vacuum tubing to the flask. Turn the vacuum on.
- 6 Using a clip, secure a glass collection cup on top of the glass funnel.
- 7 Quickly pour the entire  1 L culture into the collection cup and allow all media to filter into the bottom flask, leaving behind a layer of yeast cells on top of the filter.
- 8 Using a flamed and freshly sterilized metal spatula, quickly scrape the yeast cells off the filter.
- 9 Submerge the spatula with the scraped cells into a conical containing liquid nitrogen.
- 10 Flash freeze cells for approximately  00:01:00 -  00:02:00 and use another flamed and freshly sterilized metal spatula to scrape the cells off the first spatula and into the conical.
- 11 Screw the cap onto the conical, but do not tighten.



12 Carefully invert conical to remove excess liquid nitrogen.

13 Store frozen cells at  -80 °C until ready for use.

Note

These cells can be stored for many weeks or months in the freezer as long as they have not been lysed

Cell lysis (with Mikro-Dismembrator II)

14 Collect dry ice into a cooler.

15 Dispense liquid nitrogen into a small cryogenic bowl with a wire cage nested inside.

16 Place a clean and fully dried plastic grinding cup containing a metal ball inside the wire cage and fully submerge the grinding cup in the liquid nitrogen.

17 After approximately  00:03:00 -  00:05:00 , transfer the entire yeast sample immediately from the freezer into the cold grinding cup.

18 Submerge the grinding cup in the liquid nitrogen for  00:01:00 .

19 Using a Mikro-Dismembrator II, lyse cells at maximum speed (16 mm) for  00:01:00 .

20 Once time has elapsed, remove the grinding cup from the machine and submerge in liquid nitrogen for  00:01:00 .

21 Repeat steps 19 and 20 for a total of 10 lysis/rest cycles.  [go to step #19](#)



- 22 After lysis is complete, remove the grinding cup from the liquid nitrogen and use a flamed and freshly sterilized metal spatula to scrape the lysed cells from the cup into a conical placed on dry ice (note – record the weight of the grindate by weighing the conical before and after scraping the cells into it).
- 23 Store lysed cells at  -80 °C (grindates should be used within 24-48 hours of lysis).



Cell lysis (with CoolTeenPrep adapter)

- 24 Collect dry ice into a cooler.
- 25 Label, weigh, and record weights of empty TeenPrep Lysing Matrix A (15 mL) conicals and place on dry ice.
- 26 Transfer frozen yeast cell pellet into labelled TeenPrep conicals and return to dry ice.
- 27 Load the CoolTeenPrep adapter with dry ice (according to manufacturer's instructions).
- 28 Place sample tubes (make sure to balance the tubes inside the adapter similar to loading a centrifuge) and secure adapter in the FastPrep.
- 29 Lyse cells for  00:01:00 .
- 30 Remove tubes from adapter and let them rest on dry ice for one minute.
- 31 Repeat steps 29 and 30 for a total of 5 lysis/rest cycles.  [go to step #29](#)
- 32 After lysis is complete, weigh tubes containing samples and record weights.
- 33 Label 50 mL conicals (one per sample) and dump as much of sample/bead mixture into conical



Note

This step is optional, but it can help to avoid sample loss during resuspension with lysis buffer during **step 48 of the "Immunoprecipitation and RNA Extraction" section.**

- 34 Store lysed cells (either just the 15 mL conical or the 15 mL + 50 mL conicals if including step 33) at  -80 °C (grindates should be used within 24-48 hours of lysis).



Blocking beads for immunoprecipitation

- 35 One day prior to following the protocol in the "Immunoprecipitation and RNA extraction" section, block Pierce Anti-HA magnetic beads (ThermoFisher Scientific, #88836)



 Overnight using the steps below.

- 36 Prepare the appropriate amount of 1X lysis buffer ( 4 mL per sample; 10X lysis buffer recipe in S1 Table and 1X lysis buffer recipe in S2 Table) and 1X blocking buffer ( 0.5 mL per sample; S3 Table) needed based on the number of samples. Add water first, then the rest of the reagents in the order listed in the tables below.

A	B
	Final Concentration
Tris-HCl, pH 7.9	200 mM
Triton X-100 (Sigma, #T8787-100ML)	4%
NP-40 (Sigma, #NP40S-100ML)	1%
Sterile MilliQ Water	up to volume

S1 Table. 10X lysis buffer (can be prepared ahead of time and stored at room temperature).

A	B
	Final Concentration
10X Lysis Buffer Stock (Table S1)	10%
NH4Cl	100 mM
Halt Protease Inhibitor (ThermoScientific, #78430)	1%
EDTA, pH 8.5	5 mM
RiboLock RNase Inhibitor (ThermoFisher, #EO0382)	25 U/mL
Sterile MilliQ Water	up to volume

S2 Table. 1X lysis buffer with EDTA included (freshly prepared for each experiment).

A	B
	Final Concentration
10X Lysis Buffer Stock (Table S1)	10%
NH4Cl	100 mM
Halt Protease Inhibitor (ThermoScientific, #78430)	1%
EDTA, pH 8.5	5 mM
RiboLock RNase Inhibitor (ThermoFisher, #EO0382)	25 U/mL
BSA	10 mg/mL
Sterile MilliQ Water	up to volume

S3 Table. 1X blocking buffer (freshly prepared for each experiment).

37 Mix the lysis and blocking buffers vigorously and then place on ice.





- 38 Add the appropriate amount of Pierce Anti-HA magnetic beads ((grindate weight calculated after lysing cells * 5) * (0.04)) to a low-retention tube for each sample.
- 39 Place tubes onto a magnetic tube rack to collect the beads on the side of the tubes for approximately  00:00:30 .
- 40 Remove the buffer from each tube, quickly resuspend the beads in each tube in  1 mL of 1X lysis buffer, and place tubes on ice.
- 41 Wash beads at  4 °C for  00:02:00 with inversion on a tube rotator. 
- 42 Repeat steps 39-41 for a total of 3 washes. [➡ go to step #39](#)
- 43 After completing 3 washes, place the tubes onto a magnetic tube rack for approximately  00:00:30 .
- 44 Remove the buffer from each tube, then resuspend beads in each tube in  0.5 mL of 1X blocking buffer.
- 45 Incubate the tubes with the beads overnight at  4 °C with inversion on a tube rotator. 

Immunoprecipitation and RNA Extraction

- 46 Prepare the appropriate amount of 
- 1X lysis buffer with EDTA ( 5 mL * grindate weight for each sample; S2 Table) for the untreated samples,
 - 1X lysis buffer without EDTA ( 5 mL * grindate weight for each sample; S4 Table) for CaCl₂, CaCl₂ + MNase, MnCl₂, and MnCl₂ + DNase I samples,
 - and 1X wash buffer ( 4 mL per sample; S5 Table).
- Add water first, then add the rest of the reagents in the order they are listed in the tables. Mix lysis and wash buffers well and place on ice.

A	B
	Final Concentration
10X Lysis Buffer Stock (Table S1)	10%
NH₄Cl	100 mM
Halt Protease Inhibitor (ThermoScientific, #78430)	1%
iboLock RNase Inhibitor (ThermoFisher, #EO0382)	25 U/mL
Sterile MilliQ Water	up to volume

S4 Table. 1X lysis buffer without EDTA (freshly prepared for each experiment).

A	B
	Final Concentration
10X Lysis Buffer Stock (Table S1)	10%
KCl	300 mM
EDTA, pH 8.5	50 mM
RiboLock RNase Inhibitor (ThermoFisher, #EO0382)	25 U/mL
BSA	2 mg/mL
Sterile MilliQ Water	up to volume

S5 Table. 1X wash buffer (freshly prepared for each experiment)

- 47 Quickly dissolve grindate in appropriate amount of 1X lysis buffer (with EDTA for the untreated samples and without EDTA for the 4 sets of samples subjected to treatment;  5 mL * grindate weight calculated after lysis) while samples are on ice.
- 48 Use a glass pipet to stir the grindate and pipet up and down to thoroughly dissolve cells in lysis buffer.



49 Repeat steps 48 and 49 to completely resuspend all samples in 1X lysis buffer while incubating on ice. 

50 For the untreated samples, proceed directly to step 52. For CaCl_2 and CaCl_2 + MNase treated samples, proceed to “ CaCl_2 and CaCl_2 + MNase Samples” section below. For MnCl_2 and MnCl_2 + DNase I treated samples, proceed to “ MnCl_2 and MnCl_2 + DNase I Samples” section below.

50.1 **CaCl_2 and CaCl_2 + MNase Samples:**



1. With samples on ice, add CaCl_2 at a final concentration of 5 mM to both the CaCl_2 and CaCl_2 + MNase treated samples and mix well.
2. Add MNase (Worthington, #LS004797) at a final concentration of 100 U/ μL to CaCl_2 + MNase treated samples only and mix well.
3. Incubate both CaCl_2 and CaCl_2 + MNase treated samples at  37 °C for  00:02:00 .
4. Add EDTA (pH 8.5) at a final concentration of 5 mM to both the CaCl_2 and CaCl_2 + MNase treated samples and mix well. Place samples on ice.
5. Proceed to step 52.

50.2 **MnCl_2 and MnCl_2 + DNase I Samples:**



1. With samples on ice, add MnCl_2 at a final concentration of 10 mM to both the MnCl_2 and MnCl_2 + DNase I treated samples and mix well.
2. Add DNase I (NEB, #M0303L) at a final concentration of 100 U/mL to the MnCl_2 + DNase I treated samples only and mix well.
3. Incubate both the MnCl_2 and MnCl_2 + DNase I treated samples on ice for  00:20:00 .
4. Add EDTA (pH 8.5) at a final concentration of 5 mM to both the MnCl_2 and MnCl_2 + DNase I treated samples and mix well. Place samples on ice.
5. Incubate both the MnCl_2 and MnCl_2 + DNase I treated samples on ice for  00:30:00 .
6. Proceed to step 52.

51 Aliquot each sample into pre-chilled low retention tubes and spin samples at



 16.873 x g, 4°C, 00:15:00



- 52 Collect the supernatant for each sample into 1 conical per sample and place on ice.
- 53 Place tubes containing the Anti-HA magnetic beads and blocking buffer (blocked overnight at  4 °C) onto the magnetic tube rack and let beads settle to the side of each tube for approximately  00:00:30 .
- 54 Remove the blocking buffer from each tube.
- 55 Resuspend the beads for each sample in  1 mL of sample lysate and transfer the beads +  1 mL lysate back to the sample conical (beads and lysate will now be in the same conical).
- 56 Repeat step 56 for each sample until the beads have been incorporated into each sample conical.
- 57 Incubate samples for  03:00:00 at  4 °C with inversion on a tube rotator.  
- 58 After three hours, remove the tubes and place on ice.
- 59 Aliquot each sample into pre-chilled low retention tubes.
- 60 Place tubes on the magnetic tube rack, allowing beads to settle to the side of each tube for approximately  00:00:30 .
- 61 Discard the supernatant from each tube and resuspend beads in  1 mL of 1X wash buffer.
- 62 Wash beads for each sample for  00:02:00 at  4 °C with inversion on a tube rotator. 
- 63 Repeat steps 61-63 for a total of four washes.  [go to step #63](#)



- 64 After the final wash, place the tubes on the magnetic tube rack and allow beads to settle to the side of each tube for approximately 00:00:30 .
- 65 Discard the wash buffer from each tube and resuspend the beads for each sample in 500 μL of TES (10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.5), 1% SDS).
- 66 Add 500 μL of phenol ((pH 4.3) Fisher, #BP1751I-400) to each tube and vortex to mix.
- 67 Centrifuge samples at 17.000 x g, 00:05:00 at room temperature.
- 68 Transfer the aqueous layer for each sample to a new tube.
- 69 Repeat steps 67-69 until 2 total phenol extractions and 2 total chloroform extractions have been completed. [go to step #67](#)
- 70 After the final chloroform extraction, transfer the aqueous layer into a new tube.
- 71 Prepare a glycobblue solution in a new tube with a 1:5 ratio of GlycoBlue Coprecipitant (Invitrogen, #AM9515) to sterile MilliQ water.
- 72 Add 10 μL of the glycobblue solution (from step 72) to each sample.
- 73 Add 1.4 mL ammonium acetate precipitation solution (1 M ammonium acetate, 95% ethanol) to each sample.
- 74 Precipitate samples at -80 °C overnight or longer (can precipitate samples for weeks or even months).

Linker ligation, enzymatic linker digestion, and zinc fragmentation

- 75 Centrifuge sample tubes at  16.873 x g, 4°C, 01:00:00 
- 76 After spin, remove tubes and discard all of the precipitation solution (taking care not to disturb the RNA pellet at the bottom of the tube).
- 77 Add  750 µL of 75% ethanol to each tube.
- 78 Centrifuge samples at  17.000 x g, 00:02:00 at room temperature. 
- 79 Repeat steps 76-78 until two total ethanol washes have been completed.
- 80 After the final wash, carefully remove the ethanol and leave the caps of the tubes open until all remaining residual ethanol has evaporated.
- 81 Dissolve RNA pellets in  11.5 µL of 10 mM Tris-HCl (pH 6.9) - only need  10 µL of sample for linker ligation reaction but will use ~  1.5 µL of sample to check the RNA concentration.
- 82 Using a nanodrop, determine the RNA concentration and quality of each sample.
- 83 Make the ligation mix without the linker (will add this after denaturing) by adding the reagents in the order they are listed in the table (S6 Table), scaling up based on the number of samples. Place the mix on ice. The sequence of the UMI linker is included in S12 Table.

	A	B
		1X
	50% PEG, MW 8000 (NEB, #B1004S)	4.8 µL
	10X T4 RNA Ligase Buffer (NEB, #B0216S)	2 µL
	20 µM UMI Linker	1 µL

A	B
RiboLock RNase Inhibitor (ThermoFisher, #E00382)	0.5 µL
Sterile MilliQ Water	0.7 µL
Total Volume	9 µL

S6 Table. Ligation mix for a single sample.

- 84 Mix ligation mix vigorously. 
- 85 Add the appropriate amount of the UMI linker to a PCR tube.
- 86 Denature the UMI linker for  00:03:00 at  80 °C , then immediately place on ice. 
- 87 After denaturing, add the entire amount of the denatured UMI linker to the ligation mix and thoroughly mix. 
- 88 Transfer each RNA sample ( 10 µL) to a new PCR tube.
- 89 Denature the RNA samples for  00:02:00 at  80 °C , then immediately place the tubes on ice or onto a chilled tube rack.
- 90 Add  9 µL of the ligation mix to each sample and mix well. 
- 91 Add  1 µL of T4 RNA Ligase 2, truncated (NEB, #M0242L) to each sample and mix well. 
- 92 Incubate samples at  25 °C for  03:00:00 . 



- 93 After 3 hours have elapsed, add  2 μL of 5' Deadenylase (NEB, #M0331S) to each sample and mix well. 
- 94 Incubate samples at  30 °C for  00:45:00 . 
- 95 Dilute samples 2.5X with sterile MilliQ water supplemented 0.6X with NEBuffer 2 (NEB, #B7002S). Therefore, for a sample volume of  22 μL in volume, add  19.8 μL of NEBuffer 2 and  13.2 μL of sterile MilliQ water. Mix well. 
- 96 Add  2 μL of RecJ_f (NEB, #M0264L) to each sample and mix well. 
- 97 Incubate samples at  37 °C for  00:45:00 . 
- 98 Add  2.2 μL of fragmentation solution (100 mM Tris-HCl (pH 6.9), 100 mM ZnCl₂) to each sample and mix well. 
- 99 Incubate samples at  70 °C for  00:16:00 . 
- 100 After fragmentation is complete, immediately place tubes on ice or onto a chilled tube rack.
- 101 Add  2.5 μL of 200 mM EDTA (pH 8.5) to each sample and mix well. 
- 102 Transfer samples to a new tube, then add  1 μL glycoblue and  360 μL of ammonium acetate precipitation solution (see recipe in "Immunoprecipitation and RNA Extraction" section) to each sample.
- 103 Precipitate samples at  -80 °C overnight or longer (can precipitate samples for weeks or even months). 

Reverse transcription and size selection

- 104 Centrifuge sample tubes at  16873 x g, 4°C, 01:00:00 
- 105 After spinning, discard the remaining ammonium acetate precipitation solution from each sample tube (being careful not to disturb the pellet at the bottom of the tube).
- 106 Add  750 µL of 75% ethanol to each sample, then centrifuge samples at  17000 x g, 00:02:00 at room temperature. 
- 107 Repeat steps 105 and 106 until a total of 2 ethanol washes have been completed. 
 [go to step #105](#)
- 108 After removing the ethanol from the second wash, leave the caps of the tubes open to allow for the residual ethanol to completely evaporate from the pellet.
- 109 Dissolve pellets in  10 µL of 10 mM Tris-HCl (pH 6.9).
- 110 Prepare the reverse transcription and the RNasin/DTT mixes by adding reagents in the order that they are listed in the tables (S7 and S8 Tables), scaling up based on the number of samples. Place the mixes on ice. The oligo sequence for the NET2 primer is included in S12 Table.

A	B
	1X
5X First Strand Buffer (Included in Superscript III Kit)	3.28 µL
10 mM each dNTPs (ThermoFisher, #R0181)	1.64 µL
10 µM NET2 Primer	0.5 µL
Total Volume	5.42 µL

S7 Table. Reverse transcription mix for a single sample.

A	B
	1X
RiboLock RNase Inhibitor (ThermoFisher, #EO0382)	0.25 µL
100 mM DTT (Included in Superscript III Kit)	0.82 µL
Sterile MilliQ Water	0.25 µL
Total Volume	1.32 µL

S8 Table. RNasin/DTT mix for a single sample.

111 Mix the reverse transcription mix vigorously, then transfer  5.4 µL to one PCR tube per sample.



112 Add the entire  10 µL of each sample to PCR tubes (one sample per tube) and mix well to incorporate samples with the reverse transcription mix.



113 Incubate samples at  65 °C for  00:05:00 .



114 Immediately place the tubes on ice or onto a chilled tube rack.

115 Mix the RNasin/DTT mix vigorously, then add  1.32 µL to each sample and mix well.



116 Add  0.82 µL of Superscript III Reverse Transcriptase (Invitrogen, #56575) to each sample and mix well.



117 Incubate samples at  45 °C for  00:30:00 .



118 Add  1.8 µL of 1 M NaOH to each sample and mix well.



119 Incubate samples at  98 °C for  00:20:00 .





- 120 After reverse transcription is complete, combine samples with  20 μL of loading dye (90% formamide, 25 μM EDTA (pH 8.5), 0.025 $\mu\text{g}/\mu\text{L}$ Bromophenol Blue).
- 121 Heat samples at  100 $^{\circ}\text{C}$ for  00:05:00 . 
- 122 Load samples (leaving an entire well blank between samples or loading only loading dye into the wells between samples) onto a 10% polyacrylamide gel and run the gel at 700V for approximately  01:00:00 .
- 123 Stain the gel with  100 mL of staining solution (SYBR Gold (Invitrogen, S11494) diluted 1:10,000 with 1X TBE) at room temperature for  00:20:00 with rotation.
- 124 Meanwhile, use a sterilized 20G needle to poke a hole in the bottom of a 0.5 mL tube and nest the tube inside of a 1.5 mL tube (prepare one nested tube inside a larger tube per sample).
- 125 After staining, image gel.
- 126 Use sterile razorblades to excise samples from the gel between 120 bp (large primer band will be located just below this) and 600 bp.
- 127 Once a sample has been excised from the gel, place it into the appropriately labelled nested tube. Repeat steps 126 and 127 for all samples.  [go to step #126](#)
- 128 Centrifuge the samples at  17.000 x g, 00:03:00 at room temperature. 
- 129 Transfer any gel pieces that remain in the nested tube into the bottom tube after the spin for each sample.
- 130 Add  500 μL of sterile MilliQ water to each sample.
- 131 Incubate samples at  -80 $^{\circ}\text{C}$ for  00:15:00 .  



132 Incubate samples at  70 °C for  00:15:00 .



133 Incubate samples at  30 °C with rotation  Overnight .



134 The next day, use a mini centrifuge to collect the gel slurry at the bottom of the tubes.

135 Transfer the gel slurry for each sample into a Costar Spin-X centrifuge tube filter (Corning, #8160).

136 Centrifuge samples at  17000 x g, 00:03:00 at room temperature.



137 Discard the gel pieces collected in the top filter for each sample.

138 Transfer the liquid sample from the bottom of the tube into a new tube for each sample.

139 Add  32 µL of 3 M NaCl to each sample.

140 Add  940 µL of 100% isopropanol to each sample.

141 Add  1 µL of glycoblu to each sample.

142 Precipitate samples at  -80 °C overnight or longer (can precipitate samples for weeks or even months).



Circularization and library amplification/preparation for sequencing

143 Centrifuge sample tubes at  16873 x g, 4°C, 01:00:00



- 144** Remove and discard the ammonium acetate precipitation solution from all samples (being careful not to disturb the pellet at the bottom of the tube).
- 145** Add  750 μL of 75% ethanol and centrifuge samples at  17000 x g, 00:02:00 at room temperature.
- 146** Repeat steps 144-145 until pellets have been washed with 75% ethanol for a total of 2 times. [↩ go to step #144](#)
- 147** After the final wash, remove the ethanol and leave the caps of the tubes open to allow for all residual ethanol to evaporate.
- 148** Dissolve the pellets in  15 μL of 10 mM Tris-OAc (pH 7.9).
- 149** Prepare the circularization mix by adding the reagents in the order they are listed in the table (S9 Table), scaling up based on the number of samples. Mix well, then place mix on ice.

A	B
	1X
10X Circularization Buffer (Included in CircLigase Kit)	2 μL
1 mM ATP (Included in CircLigase Kit)	1 μL
500 mM MnCl₂ (Included in CircLigase Kit)	1 μL
Total Volume	4 μL

S9 Table. Circularization mix for a single sample.

- 150** Add  4 μL of the circularization mix to one PCR tube per sample.
- 151** Add the entire sample ( 15 μL) to each PCR tube (one sample per tube) and mix well.

152 Add  1 μL of CircLigase ssDNA Ligase (Biosearch Technologies, #CL4111K) to each sample and mix well.

153 Incubate samples at  60 °C for  01:00:00 .



154 Add an additional  1 μL of CircLigase ssDNA Ligase to each sample and mix well.

155 Incubate samples at  60 °C for  01:00:00 .



156 Incubate samples at  80 °C for  00:10:00 .



157 Transfer circularized DNA to a new tube for storage (circDNAs are very stable and excess sample can be stored at  -20 °C long-term).

158 Prepare the phusion master mix by adding the reagents in the order they appear in the table (S10 Table), scaling up based on the number of samples. Mix well and place mix on ice. The Phusion polymerase was previously purified by our lab in-house.

	A	B
		1X
	5X HF Phusion Buffer (ThermoScientific)	16.7 μL
	10 mM each dNTPs (ThermoFisher, #R0181)	1.7 μL
	Phusion Polymerase	0.8 μL
	Sterile MilliQ Water	58.4 μL
	Total Volume	77.6 μL

S10 Table. Phusion master mix for a single sample.

159 For each sample, make a unique library amplification mix by combining 77.6 μL of phusion master mix + 0.8 μL forward primer + 0.8 μL reverse primer (see S13 Table for forward and reverse primer sequences).

160 Add 1 μL of circularized DNA of each sample to a PCR tube.

161 To each sample, add 16.7 μL of the unique library amplification mix and mix well.

162 Use a thermal cycler to amplify libraries based on the cycle provided in S11 Table (25 amplification cycles total). Set lid temperature to 105 $^{\circ}\text{C}$.

	A	B	C
	Step	Temperature	Duration
	1	98 $^{\circ}\text{C}$	0:30
	2	98 $^{\circ}\text{C}$	0:10
	3	60 $^{\circ}\text{C}$	0:10
	4	72 $^{\circ}\text{C}$	1:00
	5	GOTO step 2	24X
	6	4 $^{\circ}\text{C}$	∞

S11 Table. Library amplification cycle protocol.

163 After amplification, use PCRClean DX Beads (Aline, #C-1003-5) to prepare samples for sequencing by following the manufacturer's protocol exactly.

A	B
	Sequence
20 μM UMI Linker (IDT)	5rApp/CANNNNNNNNCTCCACGAGTCATCCGC/3ddc/

A	B
10 μM NET2 Primer (IDT)	5Phos/CTGTAGGCACCATCAATGATCGTCGGA/isp18/CAC TCA/isp18/CGTCTCTTCTGCGGATGACTCGTGGAG

S12 Table. Oligo sequences for the UMI linker and reverse transcription primer.

A	B	C
Sample	Forward	Reverse
Untreated 1	CAAGCAGAAGACGGCATAACGA GATtctgcctTCCGACGATCATTG ATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
Untreated 2	CAAGCAGAAGACGGCATAACGA GATgctcaggaTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
Untreated 3	CAAGCAGAAGACGGCATAACGA GATaggagtccTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ 1	CAAGCAGAAGACGGCATAACGA GATgctcaggaTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ 2	CAAGCAGAAGACGGCATAACGA GATaggagtccTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ 3	CAAGCAGAAGACGGCATAACGA GATcatgcctaTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ + MNase 1	CAAGCAGAAGACGGCATAACGA GATgtagagagTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ + MNase 2	CAAGCAGAAGACGGCATAACGA GATcctctctgTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
CaCl₂ + MNase 3	CAAGCAGAAGACGGCATAACGA GATagcgtagcTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
MnCl₂ 1	CAAGCAGAAGACGGCATAACGA GATgtagagagTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG
MnCl₂ 2	CAAGCAGAAGACGGCATAACGA GATcctctctgTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgcCGTCTCTTCTGCG GATGACTCG

A	B	C
MnCl2 3	CAAGCAGAAGACGGCATAACGA GATagcgtagcTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgCGTCTCTTCTGCG GATGACTCG
MnCl2 + DNase 1	CAAGCAGAAGACGGCATAACGA GATcagcctcgTCCGACGATCATT GATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgCGTCTCTTCTGCG GATGACTCG
MnCl2 + DNase 2	CAAGCAGAAGACGGCATAACGA GATgcctcttTCCGACGATCATTG ATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgCGTCTCTTCTGCG GATGACTCG
MnCl2 + DNase 3	CAAGCAGAAGACGGCATAACGA GATtcctctacTCCGACGATCATTG ATGGTGCC	AATGATACGGCGACCACCGAGATC TACACtagatcgCGTCTCTTCTGCG GATGACTCG

S13 Table. Oligo sequences for library amplification indices for each sample.

A	B
Software	Version
FastQC	0.11.7
fqtrim	0.9.7
cutadapt	3.4
Anaconda	5.3.1
STAR	2.7.1a
SAMTools	1.6
BEDTools	2.28.0
R	4.0.2
RStudio	1.3.959
dplyr	1.1.10
plyr	1.8.7
ggplot2	3.3.6
ggseqlogo	0.1
ggpubr	0.4.0
cowplot	1.1.1

	A	B
	matrixStats	0.61.0
	hexbin	1.28.2
	tweedie	2.3.5
	statmod	1.4.36
	magrittr	2.0.3
	scales	1.2.1
	tidyr	1.2.1
	1.2.1	1.8-11
	DiffLogo	2.14.0
	rclone	1.48.0
	RcppRoll	0.3.0

S14 Table. R packages and versions used in data analysis.