

## **PAL-seq Protocol**

**Citation: Subtelny et al., *Nature* (2014)**

This protocol describes how to perform poly(A)-tail profiling by sequencing (PAL-seq). It is advisable to start with  $\geq 10$   $\mu\text{g}$  of total RNA, although as little as 1  $\mu\text{g}$  will yield enough final cDNA product to load an Illumina flow cell and obtain an adequate number of clusters. Prior to the first step of PAL-seq, the RNA sample should be spiked with a cocktail of poly(A)-tail length standards and, if desired, an internally radiolabeled marker RNA that is useful for tracking the efficiency of the first several steps. Sequencing should be performed using the Illumina gDNA sequencing primer, such that sequencing occurs in the 3' to 5' direction with respect to the starting RNA.

### Oligonucleotides:

3p\_biotin\_adapter: *a DNA oligo with a 5' phosphate and a 3' biotin*

Phos.AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGACACATAC.Biotin

3p\_biotin\_splint: *a DNA oligo*

TTCCGATCTTTTTTTTT

5p\_adapter: *a DNA/RNA chimera with a 5' C3 spacer*

C3spacer.CAAGCAGAAGACGGCATAACGAGTTCAGAGTTCTArCrArGrUrCrCrGrArCrGrArUrC

solexa\_rt\_primer: *a DNA oligo*

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG

solexa\_pcr\_fwd: *a DNA oligo*

CAAGCAGAAGACGGCATAACGAGTTCAGAGTTCTACAGTCCGA

solexa\_pcr\_rev: *a DNA oligo*

AATGATACGGCGACCACCGA

Step 1: Polyadenylated end capture by splinted ligation

Materials:

Total RNA (~1–50 µg)  
 Poly(A)-tail length standard mixes (~0.5 ng of each mix per µg total RNA)  
 Internally labeled marker RNA (~1000 cpm by Geiger counter)  
 3p\_biotin\_adapter (50 µM)  
 3p\_biotin\_splint (50 µM)  
 ATP (10 mM)  
 10X RNA ligase 2 buffer (NEB, B0239S) [500 mM Tris-HCl, 20 mM MgCl<sub>2</sub>, 10 mM DTT, 4 mM ATP, pH 7.5 at 25°C]  
 MgCl<sub>2</sub> (100 mM)  
 Nuclease-free water (Ambion, AM9937)  
 RNasin Plus (Promega, N261B, 40 U/µl)  
 T4 RNA ligase 2 (NEB, M0239S, 10 U/µl)  
 2X formamide loading buffer [95% formamide, 18 mM EDTA, 0.025% SDS, dyes]

Procedure:

i) Mix (in a 1.5 mL microcentrifuge tube):

Nuclease-free water	5.8 (can vary this volume as appropriate)
50 µM 3p_biotin_adapter	1.92
50 µM 3p_biotin_splint	1.6
10 mM ATP	1.28
10X RNA ligase 2 buffer	6.4
100 mM MgCl <sub>2</sub>	6.4
RNasin Plus	1.6
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	25 µl

*Room temperature, 5 min*

ii) Add:

Nuclease-free water	35.8 – (x + y + z)
Total RNA	x
Poly(A)-tail length standards	y
Marker RNA	z
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	60.8 µl

*Room temperature, 5 min*

*Remove 2.5 µl, mix with 2.5 µl 2X formamide loading buffer, save for gel analysis*

T4 RNA ligase 2	3.2
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	64 µl

*18°C, overnight (~16 h)*

## Step 2a: Partial digestion with RNase T1

Before proceeding with RNase digestion, it is recommended to check the efficiency of the previous splinted ligation step. Remove 2.5  $\mu$ l of the splinted ligation reaction and mix with 2.5  $\mu$ l 2X formamide loading buffer. Run the pre- and post-ligation samples on a 10% denaturing polyacrylamide gel and quantify the efficiency of the reaction by phosphorimaging. If necessary, supplement the splinted ligation reaction with extra T4 Rnl2 and allow it to proceed for longer.

We have had good results with the amount of RNase T1 and digestion time given below, even when variable amounts of total RNA (~1-50  $\mu$ g) are present in the reaction. If desired, optimization can be performed with an in vitro transcribed, end-labeled RNA (preferably  $\geq$ 200 nt), as described in the protocol for 3P-seq (Jan et al., 2011). Reaction conditions should be adjusted such that most of the RNA has been cut but that the fraction of fragments <36 nt (not including the 3p\_biotin\_adapter) is low. The extent of RNase T1 digestion for a PAL-seq sample can be monitored with the internally labeled marker RNA (added during the splinted ligation) by gel analysis in Step 2b.

### Materials:

EDTA (0.1 M)

GlycoBlue (Life Technologies, AM9516)

1X RNase T1 buffer (20 mM sodium citrate pH 5.0, 7 M urea, 1 mM EDTA)

RNase T1 (Life Technologies, AM2283, 1 U/ $\mu$ l)

25:24:1 Phenol:chloroform:isoamyl alcohol with 10 mM Tris and 1 mM EDTA, pH 8.0 (Sigma, P2069)

Chloroform

Precipitation/inactivation buffer (supplied with AM2283; add 3.2 mL 100% ethanol to 4.8 mL buffer before use)

2X formamide loading buffer [95% formamide, 18 mM EDTA, 0.025% SDS, dyes]

### Procedure:

i) To the splinted ligation reaction add:

0.1 M EDTA	19.2
GlycoBlue	2.4
1X RNase T1 buffer	<u>222</u>
	302.6 $\mu$ l

ii) Incubate the reaction at 50°C for 5 minutes on a heated metal block. Then remove the metal block from the heater and allow the reaction to slow cool to room temperature.

iii) Add:

RNase T1	0.6
	303.2 $\mu$ l

*Room temperature, 30 min*

iv) Extract once with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol, and again with an equal volume of chloroform.

v) To the aqueous phase, add 880  $\mu$ l of Ambion precipitation/inactivation buffer, vortex, and store at  $-20^{\circ}\text{C}$  for at least 2.5 h.

### Step 2b: Size selection

The purpose of this step is to separate splinted ligation products from unreacted 3p\_biotin\_adapter, which would otherwise form a highly abundant dimer with the 5p\_adapter during the 5' adapter ligation step.

Materials:

Ethanol

Nuclease-free water (Life Technologies, AM9937)

2X formamide loading buffer [95% formamide, 18 mM EDTA, 0.025% SDS, dyes]

Radiolabeled Decade and Century Plus ladders (Life Technologies, AM7778 and AM7145)

NaCl (0.3 M)

SUPERase•In (Life Technologies, AM2696, 20 U/ $\mu$ l)

Spin-X centrifuge tube filters (Costar, 8160)

Procedure:

i) Spin the ethanol precipitation of the RNase T1-digested RNA in a tabletop microcentrifuge at maximum speed (13,000–15,000 rpm) for 30 minutes at  $4^{\circ}\text{C}$ .

ii) Wash the resulting pellet with 70% ethanol, and air-dry at room temperature.

iii) Resuspend the pellet in 15  $\mu$ l nuclease-free water plus 15  $\mu$ l 2X formamide loading buffer.

iv) Purify the RNA on a medium-thick (0.75 mm), 20 cm long, denaturing 6% polyacrylamide gel (4W for 15 min, 12W for 15 min), running radiolabelled Decade and Century Plus ladders as size markers in adjacent lanes.

v) Size select 104–750 nt.

vi) Macerate the excised gel piece as follows: place the piece inside a 0.5 mL microcentrifuge tube that contains a hole at the bottom that was created using a red-hot 20-gauge needle. Place the 0.5 mL tube inside a 1.5 mL microcentrifuge tube, and spin the slice through the hole into the 1.5 mL tube at 20,000g for 3 min.

vii) To the macerated gel slice, add 880  $\mu$ l 0.3 M NaCl plus 8.8  $\mu$ l SUPERase•In (per ~200  $\mu$ l gel volume). Vortex and elute the RNA overnight on a rotator at room temperature.

viii) Spin the gel-eluate slurry (in ~550  $\mu$ l aliquots) through a Spin-X filter into a fresh G tube\* at 10,000g for 10 min.

ix) To each aliquot of flow-through, add 0.75  $\mu$ l GlycoBlue plus 1 mL ethanol, vortex, and precipitate at  $-20^{\circ}\text{C}$  for at least 2.5 h.

x) Shortly before starting Step 3, spin the ethanol precipitation of the size-selected RNA at maximum speed for 30 minutes at  $4^{\circ}\text{C}$ .

xi) Wash the resulting pellet with 1 ml 70% ethanol and air-dry at room temperature.

xii) Resuspend the pellet in 200  $\mu$ l nuclease-free water.

*\*Remove the filter unit of a Spin-X centrifuge tube filter from the 2.0 mL tube that houses it and place the filter unit inside a siliconized, nuclease-free 1.5 mL microcentrifuge tube. Discard the 2.0 mL tube.*

### Step 3: Capture of splinted ligation products on streptavidin beads

This step is based on the biotin capture step of 3P-seq.

#### Materials:

Bead prep solution [0.1 M NaOH, 50 mM NaCl]

NaCl (0.1 M)

2X B&W buffer [10 mM Tris-Cl pH 7.5, 1 mM EDTA, 2 M NaCl]

Wash buffer [10 mM Tris-Cl pH 7.5, 1 mM EDTA, 50 mM NaCl] (*pre-warm to 50°C*)

Dynabeads M-280 Streptavidin (Life Technologies, 11205D)

Magnet (e.g. DynaMag-2, Life Technologies, 12321D)

10X T4 PNK buffer (NEB, B0201S) [700 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 50 mM DTT, pH 7.6 at 25°C] (*dilute to 1X with water*)

#### Procedure:

- i) Aliquot 100 µl Dynabeads and magnetize for 1 min.
- ii) Remove supernatant and wash beads twice by resuspending in 100 µl bead prep solution and rotating for 2 min at room temperature.
- iii) Wash beads twice with 100 µl 0.1 M NaCl.
- iv) Wash beads with 200 µl 2X B&W buffer.
- v) Resuspend beads in 200 µl 2X B&W buffer and add RNA from previous step (in 200 µl water).
- vi) Rotate at room temperature for 15 mins.
- vii) Very briefly spin down beads (to collect them at bottom of tube), magnetize and remove supernatant. Wash beads with 400 µl 1X B&W buffer.
- viii) Wash beads twice with 400 µl wash buffer at 50°C for 2 min. After the first minute of each wash, gently vortex the tube to mix.
- ix) Wash beads twice with 400 µl 1X PNK buffer.

Step 4: 5' phosphorylation (immediately follows Step 3)

Materials:

ATP (10 mM)

10X T4 PNK buffer (NEB, B0236S) [700 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 50 mM DTT, pH 7.6 at 25°C]

Nuclease-free water (Life Technologies, AM9937)

SUPERase•In (Life Technologies, AM2696, 20 U/μl)

T4 PNK (3' phosphatase minus) (NEB, M0236S, 10 U/μl)

10X ligase buffer [500 mM Tris-HCl pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT] (*dilute to 1X with water*)

Procedure:

i) Mix (in a fresh tube):

Nuclease-free water	70
10 mM ATP	10
10X PNK buffer	10
SUPERase•In	5
<u>PNK (3' phosphatase minus)</u>	<u>5</u>
	100 μl

ii) After washing the beads from Step 3 a second time with 1X PNK buffer, magnetize and remove the supernatant.

iii) Resuspend the beads in the reaction mix from i) (at this point, I like to transfer the reaction mix plus beads to a fresh tube).

iv) Incubate the reaction at 37°C on a rotator for 1 h.

v) Magnetize the beads and remove the supernatant.

vi) Wash the beads three times with 100 μl 1X ligase buffer.

Step 5: 5' adaptor ligation (immediately follows Step 4)

Materials:

5p\_adapter (200  $\mu$ M)  
10X ligase buffer [500 mM Tris-HCl pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT]  
ATP (10 mM)  
Nuclease-free water (Life Technologies, AM9937)  
SUPERase•In (Life Technologies, AM2696, 10 U/ $\mu$ l)  
T4 RNA ligase 1 (NEB, M0204S, 10 U/ $\mu$ l)

Procedure:

i) Mix (in a fresh tube):

Nuclease-free water	14.3
200 $\mu$ M 5p_adapter	3.25
10X ligase buffer	2.5
10 mM ATP	1.2
SUPERase•In	1.25
T4 RNA ligase 1	2.5
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	25 $\mu$ l

ii) After washing the beads from Step 4 a third time with 1X ligase buffer, magnetize and remove the supernatant.

iii) Resuspend the beads in the reaction mix from i) (at this point, I like to transfer the reaction mix plus beads to a fresh tube).

iv) Incubate the reaction at room temperature on a rotator overnight (~16 h).

Step 6a: Reverse transcription (*immediately follows Step 5*)

Materials:

solexa\_rt\_primer (200  $\mu$ M)  
Nuclease-free water (Life Technologies, AM9937)  
SuperScript II (Invitrogen, 18064-022, 200 U/ $\mu$ l)  
5X First Strand buffer (supplied with SuperScript II)  
10x dNTPs (2 mM each dNTP)  
DTT (0.1 M, supplied with SuperScript II)  
NaOH (1 M)  
HEPES-NaOH pH 7 (1 M)  
NaCl (5 M)  
GlycoBlue (Life Technologies, AM9516)  
Ethanol

Procedure:

- i) Bring the 5' adaptor ligation from Step 5 to 100  $\mu$ l with nuclease-free water.
- ii) Wash the beads three times with 100  $\mu$ l nuclease-free water.
- iii) Resuspend the beads in the following mix and transfer to a PCR tube:

Nuclease-free water	29
<u>200 <math>\mu</math>M solexa rt primer</u>	<u>1</u>
	30 $\mu$ l

*65°C, 5 min*

iv) Add:

5X First Strand buffer	12.8
0.1 M DTT	6
<u>10X dNTPs</u>	<u>14</u>
	62.8 $\mu$ l

*42°C, 3 min*

v) Remove 6  $\mu$ l of the reaction and save as a minus-reverse transcriptase control.

vi) Add 2  $\mu$ l SuperScript II to the remaining bead-reaction mix.

*42°C, 1 h*

*48°C, 30 min*

vii) Add 10  $\mu$ l 1 M NaOH.

*90°C, 10 min*

vii) Transfer the bead-reaction mix to a fresh 1.5 mL microcentrifuge tube, magnetize the beads, and transfer the supernatant to another fresh 1.5 mL microcentrifuge tube.

viii) To the supernatant, add:

1 M HEPES-NaOH pH 7	50
5 M NaCl	6
GlycoBlue	1

ix) Mix well by vortexing, add 300  $\mu$ l ethanol, vortex again, and precipitate at –20°C for at least 2.5 h.

#### Step 6b: Size selection

The purpose of this step is to separate reverse transcription products containing polyadenylated end sequences from unreacted solexa\_rt\_primer and from cDNA corresponding to 5'-3' adapter dimer. (Although the gel purification following RNase T1 digestion is intended to minimize the formation of adapter dimer by size selecting a range above where unreacted 3p\_biotin\_adapter runs, some 3p\_biotin\_adapter smears upward in the gel and thus carries over into subsequent steps).

Materials:

Ethanol

Nuclease-free water (Life Technologies, AM9937)

2X formamide loading buffer [95% formamide, 18 mM EDTA, 0.025% SDS, dyes]

Radiolabeled Decade and Century Plus ladders (Life Technologies, AM7778 and AM7145)

NaCl (0.3 M)

GlycoBlue (Life Technologies, AM9516)

Spin-X centrifuge tube filters (Costar, 8160)

Procedure:

i) Spin the ethanol precipitation of the reverse transcription at maximum speed for 30 minutes at 4°C.

ii) Wash the resulting pellet with 70% ethanol, and air-dry at room temperature.

- iii) Resuspend the pellet in 3.5  $\mu$ l nuclease-free water plus 3.5  $\mu$ l 2X formamide loading buffer.
- iv) Purify the cDNA on a thin (0.35 mm), 20 cm long, denaturing 6% polyacrylamide gel (2W for 15 min, 6W for 15 min), running radiolabelled Decade and Century Plus ladders as size markers in adjacent lanes.
- v) Size select 166–790 nt.
- vi) Macerate the excised gel piece as described in Step 2b.
- vii) To the macerated gel slice, add 440  $\mu$ l 0.3 M NaCl (per  $\sim$ 100  $\mu$ l gel volume). Vortex and elute the cDNA overnight on a rotator at room temperature.
- viii) Elute for an additional 1 h at 50°C on a heating block, gently pipetting the gel-eluate slurry after 30 min to mix.
- ix) Spin the gel-eluate slurry (in  $\sim$ 500  $\mu$ l aliquots) through a Spin-X filter into a fresh G tube\* at 10,000g for 10 min.
- ix) To each aliquot of flow-through, add 0.75  $\mu$ l GlycoBlue plus 1 mL ethanol, vortex, and precipitate at  $-20^{\circ}\text{C}$  for at least 2.5 h.
- x) Spin the ethanol precipitation of the size-selected cDNA at maximum speed for 30 minutes at 4°C.
- xi) Wash the resulting pellet with 1 ml 70% ethanol and air-dry at room temperature.
- xii) Resuspend the pellet in 20  $\mu$ l nuclease-free water. Store at 4°C until further use.

*\*See note regarding this procedure in Step 2b.*

### Step 7a: PCR amplification

It is preferable to directly use the gel-purified cDNA from Step 6b for generating clusters on a flow cell instead of using PCR product derived from this cDNA. As little as 0.15 fmol of cDNA per lane can yield ~10–15 million clusters. Even the most sensitive methods for directly visualizing nucleic acids (e.g. BioAnalyzer chip) will probably not be able to detect the low amount of cDNA obtained at the end of a typical library prep, and so it is recommended to use a combination of qPCR and normal PCR to determine the concentration of the cDNA. The real-time qPCR fluorescence signal (normalized to a standard) is used to quantify concentration in terms of ng/ $\mu$ l, from which molarity can be calculated after using BioA analysis of the normal PCR product to determine the size range.

Since the cDNA is a complex mixture of sequences, over-amplification should be avoided during the normal PCR. Over-amplification refers to the stage of a PCR where repeated cycles of denaturation–reannealing occur without generation of new DNA. As a result, each molecule loses its reverse-complement partner and exists mostly as a heteroduplex paired at the adapter sequences but largely unpaired in between. Therefore, before generating PCR product for size determination, the optimal number of cycles should be determined first by performing a PCR timecourse. Every  $x$  cycles (over some range, e.g. 12–20 cycles),  $y$   $\mu$ l of a  $z$   $\mu$ l PCR are withdrawn and run on an agarose gel; this gives a sense of the size and abundance of the cDNA. Overamplification manifests as an upward shift in the product smear compared to preceding cycles, as partially single-stranded DNA runs more slowly than double-stranded DNA. A reasonable number of cycles is anywhere from the first cycle number at which product is visible on a gel to 3–4 cycles before overamplification occurs, *minus  $\log_2(z/y)$  cycles (since only a fraction of the PCR is removed and analyzed for each timepoint in the PCR timecourse)*. So, if 12 cycles is the optimal number from the timecourse, and you removed 8  $\mu$ l of a 100  $\mu$ l PCR at each timepoint (which is roughly 1/8 of the total reaction volume), perform 9 cycles when making PCR product for BioA analysis.

#### Materials:

- 10X Titanium Taq PCR buffer (Clontech, supplied with enzyme)
- 10X dNTPs (2 mM each dNTP)
- solexa\_pcr\_fwd (20  $\mu$ M)
- solexa\_pcr\_rev (20  $\mu$ M)
- Nuclease-free water (Life Technologies, AM9937)
- Titanium Taq DNA polymerase (Clontech, 639208, 50X)
- NaCl (5 M and 0.3 M)
- 6X agarose gel loading buffer [20 mM Tris-Cl pH 8.0, 15% Ficoll, 66 mM EDTA, Orange G]
- Ethanol

Procedure:

i) Mix (in a thin-walled PCR tube):

10X Titanium Taq PCR buffer	10
10X dNTPs	10
20 $\mu$ M solexa_pcr_fwd	1
20 $\mu$ M solexa_pcr_rev	1
Nuclease-free water	75
Titanium Taq DNA polymerase	2
Gel-purified library cDNA	1
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	100 $\mu$ l

ii) Thermal cycling:

1. 98°C                    1 min
2. 94°C                    30 s
3. 57°C                    1.5 min
4. Return to step 2,  $n - 1$  more times
5. 57°C                    10 min

*NOTE: Before preparing PCR product for size analysis, a PCR timecourse should be performed to determine the optimal number of cycles ( $n$ ) using the same setup as described in steps i-ii. If starting PAL-seq with 1–50  $\mu$ g total RNA, a reasonable timecourse would involve taking aliquots of the PCR every two cycles between cycles 12–20. Just before the end of the combined annealing-extension step (1.5 min at 57°C), withdraw 8  $\mu$ l of the PCR and mix with 3  $\mu$ l 6X agarose gel loading buffer. Run each timepoint on a 1.5% agarose gel. Choose  $n$  based on the guidelines described on the previous page.*

iii) To the PCR add 6  $\mu$ l 5 M NaCl, vortex, add 250  $\mu$ l ethanol, vortex again, and precipitate DNA at  $-20^{\circ}\text{C}$  for at least 2.5 h.

iv) Spin the ethanol precipitation of the PCR product at maximum speed for 30 minutes at  $4^{\circ}\text{C}$ .

v) Wash the resulting pellet with 70% ethanol, and air-dry at room temperature.

vi) Resuspend the pellet in 5  $\mu$ l 0.3 M NaCl and then add 2.5  $\mu$ l 6X agarose gel loading buffer. Store the resuspended DNA at  $4^{\circ}\text{C}$  or proceed to the next step.

Step 7b: Size selection

Materials:

NuSieve GTG agarose (Lonza, 50080)

1X TAE buffer [40 mM Tris pH 8.0, 20 mM acetate, 1 mM EDTA]  
1 kb Plus DNA ladder (Life Technologies, 10787-018)  
SYBR Gold stain (Life Technologies, S-11494)  
QIAquick Gel Extraction Kit (Qiagen, 28704)  
EB buffer [10 mM Tris-Cl, pH 8.5]

Procedure:

- i) Run the resuspended PCR product from Step 7a on a 0.8–1 cm thick, 2% NuSieve GTG agarose gel buffered with 1X TAE. In the wells adjacent to the well containing sample, load 1 kb Plus DNA ladder supplemented with bromophenol blue dye. Run the gel until the bromophenol blue reaches ~1.5 cm from the well.
- ii) Transfer the gel to a clean Petri dish (it may be necessary to first cut off the parts of the gel that do not contain sample or ladder). Stain with SYBR Gold (20,000X dilution in 1X TAE) for 1 h at room temperature with gentle shaking.
- iii) Size select 150–850 bp.
- iv) Extract the size-selected PCR product using a QIAquick Gel Extraction kit according to the manufacturer's instructions. In the final step (where DNA is eluted from the spin column), use 30  $\mu$ l EB buffer as the eluant and wait 1 minute before spinning.
- v) The sample can now be run on a BioAnalyzer chip to determine the average size of the PCR product.

Step 8: Cluster generation

Once the concentration of the gel-purified cDNA from Step 6b is known, it is possible to load a flow cell. Cluster generation is performed using a cBot, which is supplied with hybridization mix containing cDNA diluted to 1–1.5 pM. To generate the hybridization mix, we denature the cDNA with NaOH and then neutralize the reaction by adding HT1 hybridization buffer (from Illumina). We scale both the final mix volume and the NaOH concentration during denaturation to the starting cDNA concentration. For example, if a sample is very dilute (~10 pM), we make the minimum volume of hybridization mix required by the cBot (125  $\mu$ l) and perform the hybridization with the highest concentration of NaOH that can be completely neutralized by the HT1 (5 mM for a 15  $\mu$ l denaturation reaction, with 110  $\mu$ l HT1 added subsequently; this scenario is used in the protocol below). If the sample is more concentrated, then a higher concentration of NaOH can be used in the denaturation step (up to 100 mM), with correspondingly more HT1 added afterwards to neutralize. Once the hybridization mix has been made, we follow the standard Illumina cluster generation program on the cBot.

## Materials:

NaOH (2N provided by Illumina as solution HP3, 11324596) [*dilute as needed*]  
 Nuclease-free water (Life Technologies, AM9937)  
 HT1 buffer (Illumina, 15009740)

## Procedure:

## i) Mix:

NaOH (20 mM)	3.75	
Nuclease-free water	11.25 – x	
<u>Gel-purified library cDNA</u>	<u>x</u>	(add such that the final [cDNA] in the hyb mix made in the next step is 1–1.5 pM)
	15 $\mu$ l	

*Room temperature, 5 min*

## ii) Add:

<u>HT1 buffer</u>	<u>110</u>
	125 $\mu$ l

*NOTE: If you wish to use a higher concentration of NaOH in the denaturation reaction from that used above, step ii can be modified according to the following formula. Add a volume of HT1 buffer (in  $\mu$ l) equal to  $1.66 \times (\text{number of nanomoles of NaOH}) - \text{volume of the denaturation reaction}$ . (Increasing the [NaOH] requires you to increase the volume of HT1 buffer added to neutralize. Therefore,*

*using a higher [NaOH] is recommended only when the starting cDNA concentration is sufficiently high such that the final concentration of cDNA in the hyb mix will still be 1–1.5 pM). Moreover, if your starting cDNA is concentrated and you do not want to make an excessive quantity of hyb mix, you may wish to use only a fraction of the denaturation reaction when making the hyb mix. (Note that the cBot only uses 125 µl of hyb mix for cluster generation).*

iii) Pipet 125 µl of hybridization mix into a tube of a PCR 8-tube strip. (Load the other tubes with hybridization mixes made with different samples. Leftover hybridization mix can be stored at 4°C). Load the 8-tube strip into a cBot, and generate clusters on an Illumina GAII flow cell using the standard cluster generation protocol for this platform. After linearization and blocking, hybridize the Illumina gDNA sequencing primer.

### Step 9: Sequencing primer extension

In this step, the sequencing primer that was hybridized to the 3' adapter sequence in Step 8, part iii is extended through the length of the poly(A) tract with a mixture of dTTP and biotin-dUTP. This reaction is performed on an Illumina Cluster Station. (In principle, a different fluidics device could be used, as long as the flow rate and the temperature of the flow cell can be controlled, and tubes containing reagents can be easily exchanged). For the subsequent sequencing-by-synthesis step to succeed, the primer extension must proceed as far as the very first nucleotide of the poly(A) tract and no farther for every template molecule. (We have found that under some conditions, DNA polymerases add extra nucleotides opposite the poly(A)-proximal sequence after extending through the poly(A) tract). We have had the most success with Klenow fragment and low concentrations of dTTP (200 nM), replenishing the reaction mix at regular intervals so as to make sure that the [dTTP] remains constant. The reaction is carried out for 30 min so as to make sure that equilibrium is reached (shorter extension times may be possible).

The ratio of biotin-dUTP to dTTP must be tuned so that after fluorophore-tagged streptavidin is flowed on following sequencing, the fluorescence intensities of the clusters fall within the dynamic range of the sequencer. If using Alexa Fluor 532-tagged streptavidin (Life Technologies, S11224), as we do, a 1:50 mixture of biotin-dUTP:dTTP yields good results for most metazoan samples. For samples with short poly(A)-tail lengths (e.g. yeasts), the doping ratio should be increased to 1:20.

#### Materials:

2X Klenow buffer [100 mM NaCl, 20 mM Tris-HCl, 20 mM MgCl<sub>2</sub>, 2 mM DTT, 0.02% Tween, pH 7.9] (*make by supplementing NEBuffer 2 concentrate with Tween*)

dTTP (Amersham Pharmacia, 272080)

biotin-16-dUTP (Roche, 11093070910)

Klenow fragment (NEB, M0210L, 5 U/μl)

Nuclease-free water (Life Technologies, AM9937)

HT2 buffer (Illumina, 15014269) (*supplement with EDTA to 10 mM*)

HT1 buffer (Illumina, 15009740)

#### Procedure:

*NOTE: To carry out the primer extension on the Cluster Station, we run an XML script in the Cluster Station software (Primer\_Extension\_v9.xml, available at <http://bartellab.wi.mit.edu/publication.html> under "Supplemental Material" for Subtelny et al., 2014). The steps of this script are summarized below.*

- i) Prepare the reaction mix:

2X Klenow buffer	4500
1:50 biotin-16-dUTP:dTTP (100 $\mu$ M)	18 (alter doping ratio as needed)
Nuclease-free water	4302
Klenow fragment	180
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	9000 $\mu$ l

- ii) Set the flow cell temperature to 20°C.
- iii) Flow 100  $\mu$ l of 1X Klenow buffer onto each lane of the flow cell.
- iv) Flow in 100  $\mu$ l of reaction mix.
- v) Set the flow cell temperature to 37°C. Wait 2 min.
- vi) Flow in 120  $\mu$ l of fresh reaction mix. Wait 2 min.
- vii) Flow in 50  $\mu$ l of fresh reaction mix. Wait 2 min.
- viii) Repeat step vii, 12 times.
- ix) Set the flow cell temperature to 20°C.
- x) Flow in 75  $\mu$ l of HT2 buffer with 10 mM EDTA.
- xi) Flow in 75  $\mu$ l of HT1 buffer.
- xii) The flow cell can now be transferred to a GAI for sequencing.

## Step 10: Streptavidin flow-in

After the completion of sequencing-by-synthesis (in which the final base addition is followed by an extra cycle of cleavage), we perform two further cycles of cleavage to ensure that any residual fluorophore has been removed. Each cleavage cycle should be programmed to generate a “first base” report that reports the average cluster intensity in each base channel. If the average intensity is low and does not change between the two cleavage cycles, removal of residual fluorophore is complete. Unlike the streptavidin flow-in cycles which follow, the cleavage cycles need not be followed by imaging of the entire flow cell – imaging of representative tiles should be sufficient for assessing whether residual fluorophore has been removed.

The first flow-in should be performed with streptavidin-binding buffer only, so as to determine the background fluorescence intensity of each cluster. To perform this and subsequent flow-ins, run the Strep\_Flow\_In\_v8.3.xml script (available at <http://bartellab.wi.mit.edu/publication.html> and summarized in the “Procedure” below) in the GAI software. The next flow-ins that we typically perform are 30 and 100 nM streptavidin, followed by 100 nM streptavidin a second time. As with the extra cleavage cycles, each flow-in should yield a first base report to determine when streptavidin binding (as measured by intensity in the G and T channels) reaches saturation. This typically occurs after the 30 nM flow-in, but we perform at least one additional flow-in to make sure.

The flow cell can now be removed from the GAI. Unfortunately, we have had limited success with stripping flow cells once streptavidin has been flowed in (the multivalency of streptavidin may result in a cross-linked meshwork of primer-extension products that is tightly associated with the immobilized template molecules in a cluster).

### Materials:

Square plastic 125 ml bottles (Nalgene, 362015-0125)  
Wash buffer (1X PBS pH 7.4, 0.1% Tween) (*1X PBS is from Life Technologies, 10010023. Make 135 ml, and use 100 ml to make streptavidin-binding buffer*)  
Streptavidin-binding buffer (1X PBS pH 7.4, 0.1% Tween, 300 µg/ml BSA)  
Alexa Fluor 532 Streptavidin (Life Technologies, S11224)

### Procedure (repeat for each cycle of streptavidin flow-in):

i) In two square plastic bottles, prepare the 30 nM and 100 nM streptavidin solutions. First, dissolve the Alexa Fluor 532 Streptavidin (1 mg) in 1 ml nuclease-free water. Then add 47.5 µl and 158.4 µl of the dissolved streptavidin to 30 ml streptavidin-binding buffer to generate the 30 and 100 nM solutions, respectively.

- ii) To two other square plastic bottles, add 30 ml of the wash buffer and streptavidin-binding buffer.
- iii) Set the flow cell temperature to 20°C.
- iv) Flow 200  $\mu$ l of wash buffer onto each lane. We flow in non-standard reagents through position 2 on the GAll, which accommodates screw-on plastic bottles.
- v) Flow in 200  $\mu$ l of streptavidin-binding buffer.
- vi) Flow in 200  $\mu$ l of streptavidin-binding buffer (for the buffer-only condition) or 30 or 100 nM streptavidin solution.
- vii) Incubate for 10 min.
- viii) Flow in 200  $\mu$ l of wash buffer.
- ix) Proceed with generation of first-base report and imaging of flow cell.
- x) Repeat steps iv-ix until two 100 nM streptavidin flow-ins have been performed.