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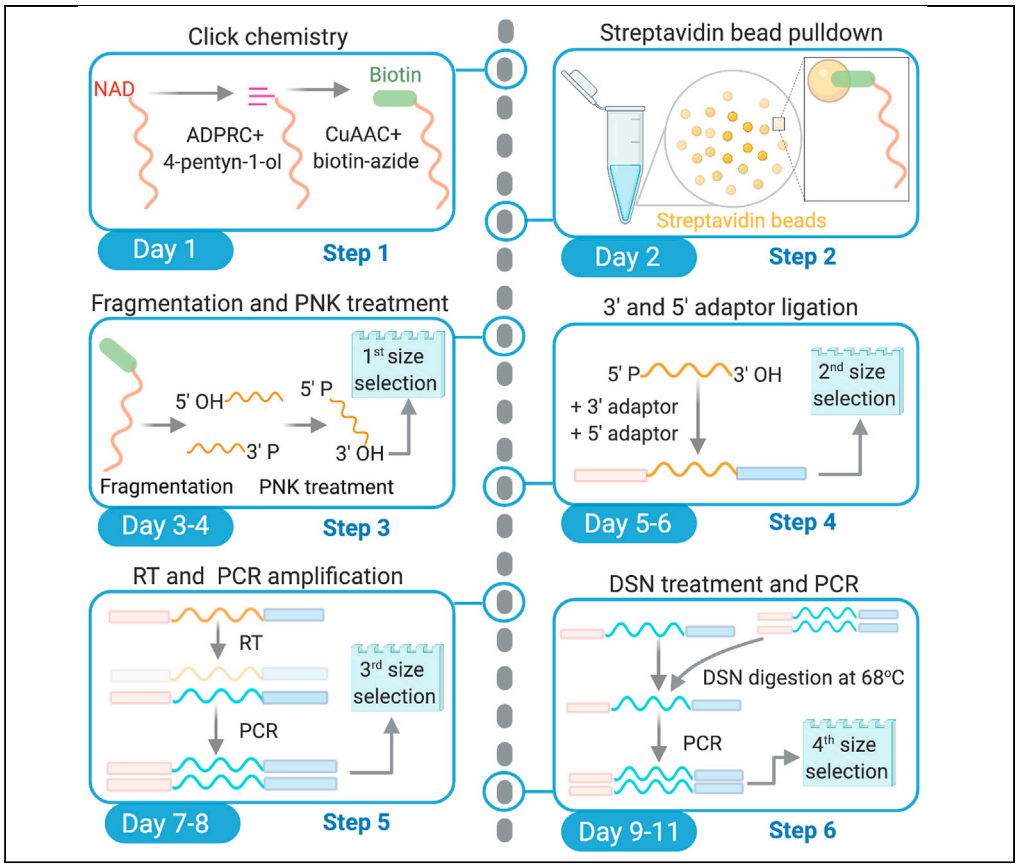
Protocol

NAD-seq for profiling the NAD$^+$ capped transcriptome of *Arabidopsis thaliana*



Eukaryotic RNAs can be modified with a non-canonical 5′ nicotinamide adenine dinucleotide (NAD$^+$) cap. NAD-seq identifies transcriptome-wide NAD$^+$ capped RNAs. NAD-seq takes advantage of click chemistry to allow the capture of NAD$^+$ capped RNAs. Unlike other approaches, NAD-seq does not require DNA synthesis on beads, but this technique utilizes full NAD$^+$ capped transcripts eluted from beads as the substrates for strand-specific RNA sequencing library preparation.

Xiang Yu, Lee E. Vandivier, Brian D. Gregory

bdgregor@sas.upenn.edu

Highlights

Protocol for NAD-seq allowing eukaryotic NAD$^+$ capped transcript discovery

Details all steps necessary for producing NAD-seq libraries

Instructions for analysis workflow to identify eukaryotic NAD$^+$ capped transcripts

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NAD-seq for profiling the NAD$^+$ capped transcriptome of *Arabidopsis thaliana*

Xiang Yu,[1,3] Lee E. Vandivier,[1,2] and Brian D. Gregory[1,2,4,*]

[1]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

[2]Cell and Molecular Biology Graduate Group, Perelman School of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

[3]Technical contact

[4]Lead contact

*Correspondence: bdgregor@sas.upenn.edu
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SUMMARY

Eukaryotic RNAs can be modified with a non-canonical 5′ nicotinamide adenine dinucleotide (NAD$^+$) cap. NAD-seq identifies transcriptome-wide NAD$^+$ capped RNAs. NAD-seq takes advantage of click chemistry to allow the capture of NAD$^+$ capped RNAs. Unlike other approaches, NAD-seq does not require DNA synthesis on beads, but this technique uses full NAD$^+$ capped transcripts eluted from beads as the substrates for strand-specific RNA sequencing library preparation.

For complete details on the use and execution of this protocol, please refer to Yu et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the reagents and specific experimental steps for using Arabidopsis wild-type Col-0 (hereafter Col-0) and *dxo1-2* mutant plant unopened flower buds for NAD-seq. We have also used this protocol on 12-day old Col-0 and *dxo1-2* mutant seedlings with and without abscisic acid (ABA) treatment. The initial input for this NAD-seq protocol is around 100 μg of total extracted RNA per sample after DNase I treatment. Although we describe the steps using Arabidopsis total RNA as the starting material, this protocol can be used on starting RNA from any eukaryotic organism (e.g., human culture cells) as long as the starting RNA concentration is high enough. For identifying NAD$^+$ capped RNAs, parallel total RNA-seq libraries using the same samples need to be prepared as background controls.

⚠ CRITICAL: All materials used in this protocol are ribonuclease (RNase)-free unless otherwise specified.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Na-HEPES	Sigma-Aldrich	Cat#H7006
ADP-ribosyl cyclase (ADPRC)	Sigma-Aldrich	Cat#A9106-1VL
Glycerol	Applied biosystems	Cat#4392215
4-pentyn-1-ol	Sigma-Aldrich	Cat#302481

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
$CuSO_4$	Sigma-Aldrich	Cat#C1297
Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)	Sigma-Aldrich	Cat#762342
Sodium ascorbate	Sigma-Aldrich	Cat#A7631
HEPES buffer, 1 M solution	Thermo Fisher Scientific	Cat# 7365-45-9
Azide-PEG_3-biiotin (biotin azide)	Jena Bioscience	Cat#CLK-AZ104P4-25
Acetylated BSA	Sigma-Aldrich	Cat#B8894
Dynabeads MyOne Streptavidin C1	Thermo Fisher Scientific	Cat#65001
RNaseOUT	Thermo Fisher Scientific	Cat#LS10777019
T4 polynucleotide kinase	New England Biolabs	Cat#M0201S
T4 DNA ligase buffer	New England Biolabs	Cat# B0202S
T4 RNA ligase 1	New England Biolabs	Cat#M0204S
T4 RNA ligase 2, truncated	New England Biolabs	Cat#M0242S
10× NEB Buffer 2	New England Biolabs	Cat#B7002S
10 mM ATP	New England Biolabs	Cat#P0756S
Duplex Specific Nuclease (DSN)	Evrogen	Cat#EA001
50 mM dNTPs (12.5 mM of each)	Promega	Cat#U1420
Sodium Acetate (3 M), pH 5.5, RNase-free	Thermo Fisher Scientific	Cat#AM9740
Glycogen	Thermo Fisher Scientific	Cat#AM9510
Phenol:chloroform	Sigma-Aldrich	Cat#77617
2-propanol	Sigma-Aldrich	Cat#34863
EDTA, 0.5 M	Sigma-Aldrich	Cat#BP2483
Formamide	Sigma-Aldrich	Cat#75-12-7
100% ethanol	Decon Labs	Cat#2716
Ultra Low Range DNA Ladder	Thermo Fisher Scientific	Cat#10597012
Gel Loading Buffer II	Thermo Fisher Scientific	Cat#AM8547
Ethidium Bromide		
Magnesium Chloride ($MgCl_2$), 1 M Solution	Affymatrix	Cat#78641
NaCl, 5 M Solution	Thermo Fisher Scientific	Cat#AM9760G
Urea	Thermo Fisher Scientific	Cat#BP169
10× TBE buffer	Thermo Fisher Scientific	Cat# 15581044
Nuclease-Free Water	Thermo Fisher Scientific	Cat#AM9937
Critical commercial assays		
RNA Fragmentation Reagents	Thermo Fisher Scientific	Cat#AM8740
SuperScript II Reverse Transcriptase	Thermo Fisher Scientific	Cat#18064014
Phusion High-Fidelity DNA Polymerase	New England Biolabs	Cat#M0531S
Deposited data		
Raw and processed NAD captureSeq (NADseq) data	This paper	GEO: GSE142390
TAIR10 Arabidopsis annotation	TAIR	ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR10_genome_release/
EPIC-CoGe genome browser	Lyons et al., 2008	https://genomevolution.org/coge/NotebookView.pl?nid=2708
Experimental models: Organisms/strains		
Arabidopsis thaliana: Col-0	ABRC	CS70000
Arabidopsis thaliana: *dxo1-2*	SALK	SALK_032903
Oligonucleotides		
TruSeq adapters (RA3 and RA5), primers and indices	Illumina	TruSeq Small RNA Sample Prep Kit
Software and algorithms		
cutadapt v1.9.1	Martin, 2011	https://cutadapt.readthedocs.io/en/stable/installation.html
STAR v2.4.2a	Dobin et al., 2013	https://github.com/alexdobin/STAR
Hisat2	Kim et al., 2019	https://daehwankimlab.github.io/hisat2/main/
HTseq v0.6.0	Anders et al., 2015	https://github.com/simon-anders/htseq

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DEseq2 v1.18.1	Love et al., 2014	https://bioconductor.org/biocLite.R
edgeR v3.13	McCarthy et al., 2012	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Other		
Fisherbrand Syringe Filters - Sterile	Thermo Fisher Scientific	Cat#09-720-3
BD 30 mL Syringe	BD	Cat#309650
15% TBE-Urea polyacrylamide gel	Invitrogen	Cat#EC6885BOX
6% TBE polyacrylamide gel	Invitrogen	Cat#EC6265BOX
Gel Breaker Tubes	IST Engineering Inc.	Cat#3388-100
Spin-X column	Sigma-Aldrich	Cat#CLS8160

MATERIALS AND EQUIPMENT

5× ADPRC reaction buffer		
Reagent	Final concentration	Amount
Na-HEPES	3.25 g	n/a
$MgCl_2$ (1 M)	25 mM	1.25 mL
H_2O	n/a	up to 50 mL
Total	**n/a**	**50 mL**

Note: This buffer can be stored at 4°C for several years.

ADPRC stock (125 μg/mL)		
Reagent	Final concentration	Amount
ADPRC	100 μg	n/a
1× ADPRC reaction buffer	n/a	400 μL
Glycerol (100%)	50%	400 μL
Total	**n/a**	**800 μL**

Note: These aliquots can be stored at −20°C for several months. Avoid freeze-thaw cycles.

50× azide-PEG3-biotin (biotin azide) stocks (100 mM solutions)		
Reagent	Final concentration	Amount
biotin azide	100 mM	25 mg
H_2O	n/a	563 μL
Total	**n/a**	**563 μL**

Note: The 1× solution is 2 mM. Aliquots can be stored at −20°C for up to 1 year. Never re-freeze thawed aliquots.

$CuSO_4$ stock (50 mM)		
Reagent	Final concentration	Amount
$CuSO_4$	50 mM	79.8 mg
H_2O	n/a	10 mL
Total	**n/a**	**10 mL**

Note: Aliquots can be stored at −20°C for up to 1 year. Avoid freeze-thaw cycles.

5× THPTA stocks (250 mM)		
Reagent	Final concentration	Amount
THPTA	250 mM	100 mg
H_2O	n/a	920 μL
Total	**n/a**	**920 μL**

Note: The 1× solution is 50 mM. Aliquots can be stored at −20°C for up to 1 year. Never re-freeze thawed aliquots.

Sodium ascorbate stock (250 mM)		
Reagent	Final concentration	Amount
Sodium ascorbate	250 mM	495.5 mg
H_2O	n/a	10 mL
Total	**n/a**	**10 mL**

Note: Aliquots can be stored at −20°C for up to several years.

Na-HEPES (100 mM)		
Reagent	Final concentration	Amount
Na-HEPES	100 mM	1.3 g
H_2O	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Note: Adjust the pH to 7.2. This buffer can be stored at 4°C for several years.

Immobilization buffer		
Reagent	Final concentration	Amount
Na-HEPES (pH 7.2) (100 mM)	10 mM	5 mL
NaCl (5 M)	1 M	10 mL
EDTA (0.5 M)	5 mM	0.5 mL
H_2O	n/a	34.5 mL
Total	**n/a**	**50 mL**

Note: This buffer can be stored at 4°C for several years.

Immobilization buffer with 1000× BSA (100 mg/mL)		
Reagent	Final concentration	Amount
acetylated BSA	100 mg/mL	100 mg
immobilization buffer	1 M	Up to 1 mL
Total	**n/a**	**1 mL**

Note: The 1× solution is Immobilization buffer with 100 μg/mL BSA. This solution needs to be made fresh for each experiment.

Streptavidin wash buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.5) (1 M)	50 mM	2.5 mL
Urea	24 g	n/a
H_2O	n/a	Up to 50 mL
Total	**n/a**	**50 mL**

Note: Dissolving the urea may require light heating. This solution can be stored at 22°C–27°C for several years. Avoid cold temperatures and freezing, as this leads to precipitation of urea. If urea crystals form, heat to dissolve urea or replace it with fresh buffer.

Elution buffer (10 mM EDTA in 95% formamide)		
Reagent	Final concentration	Amount
Formamide	95%	9.5 mL
0.5 M EDTA	10 mM	0.2 mL
H_2O	n/a	0.3 mL
Total	**n/a**	**10 mL**

⚠ CRITICAL: Formamide is toxic, wear gloves and work in a fume hood.

Note: This buffer can be stored at 4°C for several years.

Hybridization buffer		
Reagent	Final concentration	Amount
1 M HEPES buffer	200 mM	200 μL
5 M NaCl	2 M	400 μL
H_2O	n/a	400 μL
Total	**n/a**	**1 mL**

Note: This buffer can be stored at 4°C for several years.

RT solution		
Reagent	Final concentration	Amount
RNA	n/a	6 μL
RT primer (100 μM)	8 μM	1 μL
5× First Strand Buffer	1×	2 μL
dNTP mix (12.5 mM)	0.5 mM	0.5 μL
DTT (100 mM)	8 mM	1 μL
RNase Inhibitor	n/a	1 μL
SuperScript II Reverse Transcriptase	n/a	1 μL
Total	**n/a**	**12.5 μL**

Note: This solution needs to be made fresh for each experiment.

PCR solution		
Reagent	Final concentration	Amount
Phusion Mix (2×)	1×	50 μL
Betaine 5 mM	2 mM	40 μL
RNA PCR Primer (RP1) (10 μM)	0.2 μM	2 μL
RNA PCR Primer Index (RPIX)* (10 μM)	0.2 μM	2 μL
DNA	n/a	6 μL
Total	**n/a**	**100 μL**

Note: This solution needs to be made fresh for each experiment.

STEP-BY-STEP METHOD DETAILS

ADPRC reaction and copper click chemistry

Timing: 1 day

This step converts 5′ NAD^+ capped RNA into 5′ biotin-modified RNA through ADPRC treatment followed by copper click chemistry.

1. For the ADPRC reaction (per sample), incubate 50 μL of RNA with ADPRC and 4-pentyn-1-ol.
   a. In a 1.5 mL tube, add 20 μL of 5× ADPRC buffer into the 50 μL of RNA.
   b. Add 10 μL of 4-pentyn-1-ol.
   c. Add 20 μL (at least 1.5U) of ADPRC. Now the volume is 100 μL.
   d. Incubate at 37°C for 30 min in an Eppendorf Thermomixer.

2. Halt reaction through two rounds of phenol-chloroform extraction.
   a. Add 100 μL of H_20, now the volume is 200 μL.
   b. Add 200 μL of Phenol:chloroform: IAA.
   c. Vortex for 15 s.
   d. Let sit at 22°C–27°C for 2 min.
   e. Spin 12,000 rpm at 4°C for 15 min.
   f. Extract as much aqueous layer as possible (~200 μL).
   g. Repeat extraction steps b-f above.

Note: do not add more nuclease-free H_20.

   h. Precipitate RNA by adding 600 μL of 100% EtOH, 20 μL of 3M NaOAc (pH 5.5), and 3 μL of glycogen. Store at −80°C for 2 h.

Pause point: If not doing the copper click reaction in the same day, leave at −80°C overnight.

   i. Centrifuge at max speed for 40 min.
   j. Wash with 750 μL of 80% EtOH.
   k. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
   l. As soon as the pellet is dry, resuspend in 20.4 μL of 5× ADPRC buffer and 63.7 μL of Nuclease-free H_20.
   m. Allow the samples to resuspend for 20 min on ice before proceeding.

3. For copper click reaction per sample,
   a. Prepare copper mix.
      i. Add 2 μL of 50 mM $CuSO_4$.
      ii. Add 1 μL of 50 mM THPTA.
      iii. Add 0.4 μL of 250 mM Sodium ascorbate.
   b. Mix 84.1 μL of RNA with 12.5 μL of Biotin azide and 3.4 μL of copper mix made above.
   c. Vortex and incubate at 25°C, 30 min, shaking at 350 rpm using the shaker.

4. Halt reaction through two rounds of phenol-chloroform extraction.
   a. Add 100 μL of H_20, now the volume is 200 μL.
   b. Add 200 μL of Phenol:chloroform: IAA.
   c. Vortex for 15 s.
   d. Let sit at 22°C–27°C for 2 min.
   e. Spin 12,000 rpm at 4°C for 15 min.
   f. Extract as much aqueous layer as possible (~200 μL).
   g. Repeat extraction steps b-f above.

   Note: do not add more nuclease-free H_20.

   h. Precipitate RNA by adding 600 μL of 100% EtOH, 20 μL of 3M NaOAc (pH 5.5), and 3 μL of glycogen. Store at −80°C at least 1 h.

   Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

   i. Centrifuge at max speed at least 40 min.
   j. Wash with 750 μL of 80% EtOH.
   k. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
   l. As soon as the pellet is dry, resuspend in 40 μL of Immobilization buffer.
   m. Allow the samples to resuspend for 20 min on ice before proceeding.

Streptavidin bead pulldown

Timing: 1 day

The purpose of this step is to pull down 5′ biotin-modified RNA using streptavidin-coated magnetic beads. Instead of performing Reverse Transcription for cDNA synthesis on beads as done in the NAD captureSeq (Winz et al., 2017) protocol, our NAD-seq elutes full NAD^+ capped transcripts from the beads (Yu et al., 2021).

5. Prepare the beads (Dynabeads MyOne Streptavidin C1).
   a. In a single 1.5 mL tube mix enough beads for all reactions (20 μL of beads per reaction).
   b. Place on magnet to remove storage buffer.
   c. Wash 3× with 300 μL of immobilization buffer.
   d. Block with immobilization buffer containing 100 μg/mL acetylated BSA. Mix end-over-end at 22°C–27°C for 20 min using a tube rotator.
   e. Wash 3× with 300 μL of immobilization buffer.
   f. Resuspend beads in 160 μL of immobilization buffer per reaction. Aliquot beads to round-bottom 2 mL Eppendorf tubes.

   Note: 10-fold dilution of original beads once RNA is added.

6. Capture biotinylated (originally NAD^+ capped) RNA.

a. Add 40 μL of biotinylated RNA (from step 1) to beads.
b. Mix end-over-end for 1 h at 22°C–27°C using a tube rotator.
c. Remove supernatant and resuspend beads in 300 μL of streptavidin wash buffer.
d. Transfer beads to a fresh 1.5 mL Eppendorf tube.
e. Wash 3× with 300 μL of streptavidin wash buffer. Each time mix 5 min end-over-end using a tube rotator.
f. Wash 3× with nuclease-free H_20.
g. Elute by adding 50 μL of elution buffer and incubating at 65°C for 5 min.

⚠ CRITICAL: Do not quick spin the tube.

h. Immediately place on magnet and transfer supernatant to a fresh 1.5 mL Eppendorf tube.

7. Ethanol precipitate RNA. Troubleshooting 1
   a. Add 250 μL of Nuclease-free H_20 into the 50 μL of eluted RNA sample.

   ⚠ CRITICAL: Formamide must be <30% for precipitation to work.

   b. Precipitate the RNA with 30 μL of 3M NaOAc (pH 5.5), 3 μL of glycogen and 900 μL of 100% EtOH. Store at −80°C at least 1 h.

   ⏸ **Pause point:** We usually leave the samples at −80°C for 12–16 h at this stage.

   c. Centrifuge at max speed at least 40 min.
   Excepted result: After spinning, a pellet should be visible, indicating that appropriate levels of NAD^+ capped RNAs are captured.
   d. Wash the pellet with 750 μL of 80% EtOH.
   e. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
   f. As soon as the pellet is dry, resuspend in 9 μL of Nuclease-free H_2O.

   Note: the total volume for RNA fragmentation next step is 10 μL. Alternatively, resuspend the pellet in 10 μL of nuclease-free H_2O, and use 1 μL to quantify the concentration of NAD^+ capped RNAs using a Nanodrop.

   g. Allow the samples to resuspend for 20 min on ice before proceeding.

RNA fragmentation, PNK treatment, and first size selection

⏱ Timing: 2 days

The purpose of this step is to fragment the full RNA molecules into smaller fragments, convert the 5′ OH and 3′ P of fragmented RNA pieces to 5′ P and 3′ OH, and select RNA fragments within the preferred size range.

8. RNA fragmentation.
   a. Add 1 uL of 10× Fragmentation Reagent to each sample (total volume 10 μL).
   b. Incubate the reactions at 70°C for 5 min in the digital heating block.

   ⚠ CRITICAL: The reaction volume and incubation time are important for obtaining RNA fragments within the preferred size range.

   c. After 5 min, add 1 μL of stop solution to the fragmentation reaction.

d. Bring the volume up to 100 μL by adding 89 μL of nuclease-free H_2O.
e. Precipitate the fragmented RNA by adding 10 μL of 3M NaOAc (pH 5.5), 3.0 μL of glycogen, and 300 μL of 100% EtOH.
f. Freeze at least 1.5 h at −80°C.

Pause point: If not continuing to the next steps on the same day, leave at −80°C for 12–16 h.

g. Centrifuge at maximum speed for 70 min at 4°C. If only in −80°C for 1 h, spin for an extra 10 min.
h. Remove supernatant. Wash with 750 μL of 80% EtOH.
i. Centrifuge at maximum speed for 5 min at 4°C.
j. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
k. As soon as the pellet is dry, resuspend in 17 μL of nuclease-free H_2O.

Note: the total volume for PNK treatment in the next step is 20 μL.

l. Allow the samples to resuspend for 20 min on ice before proceeding.

9. PNK treatment.
   a. In 17 μL of RNA sample, add 2 μL of 10× T4 DNA ligase buffer and 1 μL of T4 polynucleotide kinase (T4 PNK).

   Note: Instead of using the T4 PNK buffer, NEB's 10× T4 DNA ligase buffer (B0202S), which contains ATP, is used in this step.

   b. Incubate the reactions at 37°C for 1 h in the digital heating block.
   c. Bring the volume up to 100 μL by adding 80 μL of nuclease-free H_2O.
   d. Precipitate the fragmented RNA by adding 10 μL of 3M NaOAc (pH 5.5), 3.0 μL of glycogen and 300 μL of 100% EtOH.
   e. Freeze at least 1.5 h at −80°C.

   Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

   f. Centrifuge at maximum speed for at least 70 min at 4°C. If only in −80°C for 1 h, spin for an extra 10 min.
   g. Remove supernatant. Wash with 750 μL of 80% EtOH.
   h. Centrifuge at maximum speed for 5 min at 4°C.
   i. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
   j. As soon as the pellet is dry, resuspend in 10 μL of Nuclease-free H_20.
   k. Allow the samples to resuspend for 20 min on ice before proceeding.

10. First size selection
    a. Put 15% TBE-urea polyacrylamide gel (10 wells) in the gel box such that the wells face inward, place a balancer opposite if running only one gel, and lock it into position. Pour about 700 mL of 1× TBE buffer into the gel box.

    Note: First filling the space between the gel and balancer and then pouring the rest on the outside.

    b. Wash the wells out 3× with 1 mL pipette using the running buffer in the gel box.
    c. Pre-run the gel for 25 min at 155 V.

d. 15 min before the pre-run is complete, prepare the ladder and samples:
   i. Prepare the ladder by mixing 1.5 μL of Ultra Low Range DNA Ladder, 8.5 μL H_20, and 10 μL of Gel Loading Buffer II in a 1.5 mL tube.
   ii. To each of the samples, add 10 μL of Gel Loading Buffer II.
   iii. Put samples (BUT NOT THE LADDER) at 70°C for 5 min and then place on ice for 3 min.
e. Immediately prior to loading the gel, wash out the wells 3×.
f. Load DNA ladder and samples into the gel.

⚠ CRITICAL: Keep a space between samples and avoid the end wells on the gel.

g. Run gel at 155 V for about 1.5 h.

Note: Purple dye runs at about 10 base pairs (bp), so stop when purple dye is ~3/4 of the way down.

h. While running, poke holes in 0.5 mL tubes using a 21 G needle and place inside 2 mL tubes.

Optional: 0.5 mL Gel Breaker Tubes that already have holes could be used as an alternative, but the size of the holes still needs to be increased by running a 21 G needle through each hole.

i. Stain the gel with ethidium bromide (EtBr). Add 14 μL of 10 mg/mL EtBr to 200 mL of 1× TBE buffer in a clean RNase-free tray. Add the gel and rock for 10 min.
j. Take a picture of the gel. Then cut the region of the gel corresponding to 50–250 nucleotides (nt) using a clean razor blade and put the gel slice in the 0.5 mL tubes.

⚠ CRITICAL: The razor blade needs to be sterilized using 100% EtOH and should be changed when cutting each sample gel lane to avoid sample cross-contamination.

k. Spin the samples at 14,000 rpm speed at 4°C for 2 min. Make sure all of the gel goes through the holes. If not, spin again for 1 min and repeat until all gel is out of 0.5 mL tube, poking more holes in the tubes if necessary.
l. Add 300 μL of 0.3M NaCl and rotate for 4 h.
m. After 4 h, pipette the entire sample into a Spin-X column, and spin at 14,000 rpm at 4°C for 2 min. Make sure all of the liquid spins out. There should be about 300 μL. Spin longer if necessary.
n. Precipitate the RNA by adding 3 μL glycogen, 30 μL 3M NaOAc (pH 5.5), and 900 μL 100% EtOH.
o. Leave at −80°C at least 1 h.

Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

p. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
q. Remove supernatant. Wash with 750 μL of 80% EtOH.
r. Centrifuge at maximum speed for 5 min at 4°C.
s. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
t. As soon as the pellet is dry, resuspend in 5 μL of Nuclease-free H_2O.

3′ and 5′ adapter ligation and second size selection

Timing: 2 days

The purpose of this step is to ligate the 3′ and 5′ adapters onto the PNK-treated RNA fragments and use gel-mediated size selection to isolate adapter-ligated RNA fragments in the proper size range while removing any potential adapter-adapter dimers.

11. 3′ adapter ligation.
    a. In 5 μL of PNK-treated RNA, add 1 μL of RA3 3′ adapter, mix, and spin.
    b. Incubate the tube in the pre-heated thermal cycler at 70°C for 2 min and then 4°C for 2 min.
    c. Prepare mix: add 2 μL of T4 RNA ligation buffer, 1 μL of RNase OUT, and 1 μL of T4 RNA Ligase 2 truncated, mix, and spin.
    d. Add 4 μL of the mix to each pre-heated sample (6 μL), mix, and spin. The total volume of the reaction should be 10 μL. Place back in thermal cycler at 28°C for 1:15 h.
12. 5′ adapter ligation.
    a. With 5 min left to go, heat 1 μL of RA5 5′ adapter (25 μM) per sample at 70°C for 2 min and then put it on ice for 2 min.
    b. Prepare mix: add 1 μL of 10 mM ATP and 1 μL of T4 RNA Ligase 1. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
    c. Add the mix into the RA5 5′ adapter. Gently pipette the entire volume up and down 6–8 times to mix thoroughly.
    d. Add 3 μL of the mix from the aliquoted RA5 5′ adapter tube to the reaction from step 2 of RA 3′ adapter-linked RNA. The total volume of the reaction should be 13 μL.
    e. Place back in thermal cycler at 28°C for 1 h.

Pause point: Place in −20°C freezer for 12–16 h. Unless proceeding directly to next step.

13. Second size selection.
    a. Put 15% TBE-urea polyacrylamide gel (10 wells) in the gel box such that the wells face inward, place a balancer opposite if running only one gel, and lock it into position. Pour about 700 mL of 1× TBE buffer into the gel box.

    Note: First filling the space between the gel and balancer and then pouring the rest on the outside.

    b. Wash the wells out 3× with 1 mL pipette using the running buffer in the gel box.
    c. Pre-run the gel for 25 min at 155 V.
    d. 15 min before the pre-run is complete, prepare the ladder and samples:
        i. Prepare the ladder by mixing 1.5 μL of Ultra Low Range DNA Ladder, 8.5 μL of H_20, and 10 μL of Gel Loading Buffer II in a 1.5 mL tube.
        ii. To each of the samples, add 10 μL of Gel Loading Buffer II.
        iii. Put samples (BUT NOT THE LADDER) at 70°C for 5 min and then place on ice for 3 min.
    e. Immediately prior to loading the gel, wash out the wells 3×.
    f. Load DNA ladder and samples into the gel.

    ⚠ CRITICAL: Keep a space between samples and avoid the end wells of the gel.

    g. Run gel at 155 V for about 1.5 h. Purple dye runs at about 10 bp, so stop when purple dye is ~3/4 of the way down.
    h. While running, poke holes in 0.5 mL tubes using a 21 G needle and place inside 2 mL tubes.

    Optional: you can use 0.5 mL Gel Breaker Tubes that already have holes, but you still need to increase the size of the holes by running a 21 G needle through each hole.

    i. Stain the gel with EtBr. Add 14 μL of 10 mg/mL EtBr to 200 mL of 1× TBE buffer in a clean RNase-free tray. Add the gel and rock for 10 min.

j. Take a picture of the gel. Then cut the region of the gel that corresponds to 100–300 nt using a clean razor blade and put the gel slice in the 0.5 mL tubes.

Note: Adapter-adapter dimers are 50 nt at this point, and the first RNA size selection is 50–250 nt, so the preferred size range in this step is 100–300 nt.

⚠ CRITICAL: The razor blade needs to be sterilized with 100% EtOH and should be changed when cutting the gel lanes corresponding to each different sample to avoid sample cross-contamination.

k. Spin the samples at 14,000 rpm, 4°C for 2 min. Make sure most/all of the gel goes through the holes. If not, spin again, poking more holes in the tubes if necessary.
l. Add 300 μL of 0.3M NaCl and rotate for 4 h.
m. After 4 h, pipette the entire sample into a Spin-X column, and spin at 14,000 rpm at 4°C for 2 min. Make sure all of the liquid spins out. There should be about 300 μL. Spin longer if necessary.
n. Precipitate the RNA by adding 3 μL of glycogen, 30 μL of 3M NaOAc (pH 5.5), and 900 μL of 100% EtOH.
o. Leave at −80°C at least 1 h.

Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

p. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
q. Remove supernatant. Wash with 750 μL of 80% EtOH.
r. Centrifuge at maximum speed for 5 min at 4°C.
s. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
t. As soon as the pellet is dry, resuspend in 6 μL of nuclease-free H_2O.

Reverse transcription, PCR amplification, and third size selection

Timing: 2 days

This step uses reverse transcription to synthesize complementary DNA of adapter-ligated RNA, amplifies these desired DNA molecules while also adding index primers to each RNA sequencing library, and uses gel-mediated size selection to isolate adapter-ligated RNA fragments in the proper size range while removing any potential adapter-adapter dimers.

14. Reverse transcription.
    a. Preheat the thermal cycler to 70°C.
    b. add 1 μL of RNA RT Primer (RTP, 100 μM) into 6 μL of 5′ and 3′ Adapter-ligated RNA.
    c. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.
    d. Incubate the tube in the preheated thermal cycler at 70°C for 2 min and then 4°C for 2 min.
    e. Prepare the following RT mix per sample:
        i. Add 2 μL of 5× First Strand Buffer.
        ii. Add 0.5 μL of 12.5 mM dNTP mix.
        iii. Add 1 μL of 100 mM DTT.
        iv. Add 1 μL of RNase Inhibitor.
        v. Add 1 μL of SuperScript II Reverse Transcriptase.
    f. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly.

g. Add 5.5 μL of the mix to the reaction tube from step b. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly. The total volume should now be 12.5 μL.
h. Incubate the tube on the thermal cycler at 50°C for 1 h.

15. PCR amplification.
    a. Set the PCR program:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	11 cycles
Annealing	60°C	30 s	
Extension	72°C	15 s	
Final extension	72°C	10 min	1
Hold	4°C	forever	

    b. Make master mix for PCR solution per sample, which is everything listed below except for the index primer.
        i. Add 35 μL of 2× Phusion Mix.
        ii. Add 21 μL of 5 mM Betaine.
        iii. Add 2 μL of 10 μM RNA PCR Primer.

Note: Make 10% extra reagent if you are preparing for multiple samples. A different PCR Index Primer is used for each sample, so add this directly to the sample rather than including in the master mix.

    c. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, then centrifuge briefly, and place the tube on ice.
    d. Add 58 μL of the PCR master mix to each sample tube. Add 2 uL of the correct 10 μM RNA PCR Index Primer.
    e. Gently pipette the entire volume up and down 6–8 times to mix thoroughly, centrifuge briefly, and place the tube on ice. The total volume should now be 72.5 μL.
    f. Aliquot the reaction into 3 PCR tubes, with approximately 25 μL in each tube.
    g. Place in the thermal cycler and run the above PCR program.
    h. Merge 3 tubes of PCR product in a 1.5 mL tube.
    i. Precipitate the DNA by adding 3 μL of glycogen, 30 μL of 3M NaOAc (pH 5.5), and 300 μL of 100% EtOH.
    j. Leave at −80°C at least 1 h.

Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

    k. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
    l. Remove supernatant. Wash with 750 μL of 80% EtOH.
    m. Centrifuge at maximum speed for 5 min at 4°C.
    n. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
    o. As soon as the pellet is dry, resuspend in 6 μL of Nuclease-free H_2O.

16. Third size selection. Troubleshooting 2

a. Put 6% TBE polyacrylamide gel (10 wells) in the gel box such that the wells face inward, place a balancer opposite if run only one gel, and lock it into position. Pour about 700 mL of 1× TBE buffer into the gel box.

Note: There is no urea in this gel, so there is no need to pre-run it.

b. Prepare the ladder and samples:
   i. Mix 1.5 μL of Ultra Low Range DNA Ladder, 8.5 μL of Nuclease-free H_2O, and 10 μL of Gel Loading Buffer II.
   ii. Add 10 μL of Gel Loading Buffer II to each of the samples.
c. Load DNA ladder and samples into the gel.

⚠ CRITICAL: Keep a space between samples and avoid the lanes at the ends of the gel.

d. Run gel at 155 V for about 30 min.

Note: Until the purple dye touches the bottom of the gel.

e. While running, poke holes in 0.5 mL tubes using a 21 G needle and place inside 2 mL tubes.

Optional: you can use 0.5 mL Gel Breaker Tubes that already have holes, but you still need to increase the size of the holes by running a 21 G needle through each hole.

f. Stain the gel with EtBr. Add 14 μL of 10 mg/mL EtBr to 200 mL of 1× TBE buffer in a clean RNase-free tray. Add the gel and rock for 7 min.
g. Take a picture of the gel. Then cut the region of the gel that corresponds to 168 bp to the end of signal using a clean razor blade and put the gel slice in the 0.5 mL tubes.
   Excepted result: The DNA signal in the gel above 168 bp should be visible, indicating the adaptor ligation and PCR amplification worked successfully.

Note: Adapter-adapter dimer is 118 bp at this point, and the first RNA size selection is 50–250 nt, so the preferred size range in this selection is 168–368 bp.

⚠ CRITICAL: The razor blade needs to be sterilized with 100% EtOH and should be changed when cutting gel lanes corresponding to the different samples to avoid sample cross-contamination.

h. Spin the samples at 14,000 rpm, 4°C for 2 min. Make sure most/all of the gel goes through the holes. If not, spin again for 1 min, poking more holes in the tubes if necessary.
i. Add 300 μL of 1× NEB Buffer 2 and rotate for 2 h.
j. After 2 h, pipette the entire sample into a Spin-X column, and spin at 14000 rpm at 4°C for 2 min. Make sure all of the liquid spins out. There should be about 300 μL. Spin longer if necessary.
k. Precipitate the DNA by adding 3 μL of glycogen, 30 μL of 3M NaOAc (pH 5.5), and 900 μL of 100% EtOH.
l. Leave at −80°C at least 2 h.

Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

m. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
n. Remove supernatant. Wash with 750 μL of 80% EtOH.
o. Centrifuge at maximum speed for 5 min at 4°C.

p. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
q. As soon as the pellet is dry, resuspend in 12 μL of Nuclease-free H_2O.

DSN treatment and PCR amplification

Timing: 3 days

This step treats the DNA library samples with Duplex Specific Nuclease (DSN) normalization to reduce high abundant DNA molecules derived from rRNA and tRNA, and then amplify the DSN-treated DNA samples through a PCR reaction.

17. DSN Treatment. Troubleshooting 4
    a. Put a total of 100 ng of sample DNA into 13.5 μL of Nuclease-free H_2O in 200 μL PCR tubes.

    Note: Store the remaining library at −80°C. The DSN treatment can be repeated if more library is necessary for sequencing.

    b. Add 4.5 μL of Hybridization buffer and mix thoroughly.
    c. Incubate samples in the thermocycler 98°C 2 min, followed by 68°C 5 h.

    Note: The 5 h incubation has been found to reduce rRNA and tRNA in Arabidopsis. For other species a range of incubation times should be tested.

    d. After 4.5 h, dilute 4 μL of 10× DSN Master buffer in 16 μL of Nuclease-free H_2O to make 2× of DSN Master buffer.
    e. Incubate 2× DSN Master buffer at 68°C for the remainder of the 5 h incubation.
    f. Add 20 μL of 2× DSN Master buffer to each sample and mix thoroughly.

    Note: Do not allow samples to cool down, mix by pipetting and briefly centrifuge.

    g. Incubate samples at 68°C for another 10 min.
    h. Add 2 μL of DSN enzyme to DNA samples and mix thoroughly.

    Note: Do not allow samples to cool down, mix by pipetting and briefly centrifuge.

    i. Incubate samples at 68°C for another 25 min.
    j. Add 40 μL of DSN STOP solution (10 mM EDTA) and mix thoroughly.
    k. Transfer samples to 1.5 mL tubes and add 20 μL of Nuclease-free H_2O, bringing the volume up to 100 μL.
    l. Precipitate the DNA by adding 3 μL of Glycogen, 10 μL of 3M NaOAc (pH 5.5), and 300 μL of 100% EtOH.
    m. Leave at −80°C at least 2 h.

    Pause point: We usually leave the samples at −80°C for 12–16 h at this stage.

    n. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
    o. Remove supernatant. Wash with 750 μL of 80% EtOH.
    p. Centrifuge at maximum speed for 5 min at 4°C.
    q. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
    r. Resuspend DNA in 6 μL of Nuclease Free H_2O. Allow samples to resuspend on ice for 25 min.

18. PCR amplification.
    a. Put the 6 μL of DSN-treated samples in new PCR tubes.
    b. Make the Phusion PCR mix, which is everything listed below except for the index primer.
        i. Add 50 μL of 2× Phusion Mix.
        ii. Add 40 μL of 5 mM Betaine.
        iii. Add 2 μL of 10 μM RNA PCR Primer.

    Note: Make 10% extra reagent if you are preparing multiple samples. A different PCR Index Primer is used for each sample, so add this directly to the sample rather than including in the master mix.

    c. Add 92 μL of the Phusion PCR mix and 2 μL of the appropriate PCR Index Primer to each DSN-treated sample.
    d. Divide each 100 μL of sample volume into 4 PCR tubes (25 μL/tube).
    e. Set the PCR program on PCR machine.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	11 cycles
Annealing	65°C	30 s	
Extension	72°C	15 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

    f. Merge the 100 μL of PCR product in a 1.5 mL tube.
    g. Precipitate the DNA by adding 3 μL of glycogen, 10 μL of 3M NaOAc (pH 5.5), and 300 μL of 100% EtOH.
    h. Leave at −80°C at least 1 h.

    ⏸ **Pause point:** If not doing the size selection the same day, leave at −80°C for 12–16 h.

    i. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
    j. Remove supernatant. Wash with 750 μL of 80% EtOH.
    k. Centrifuge at maximum speed for 5 min at 4°C.
    l. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
    m. Resuspend DNA in 10 μL of Nuclease-free H_2O. Allow samples to resuspend on ice for 25 min.

19. Forth size selection and quantification.
    a. Put 6% TBE polyacrylamide gel (10 wells) in the gel box such that the wells face inward, place a balancer opposite if run only one gel, and lock it into position. Pour about 700 mL of 1× TBE buffer into the gel box.

    Note: There is no urea in this gel, so there is no need to pre-run it.

    b. Prepare the ladder and samples:
        i. Mix 1.5 μL of Ultra Low Range DNA Ladder, 8.5 μL of Nuclease-free H_2O, and 10 μL of Gel Loading Buffer II.
        ii. Add 10 μL of Gel Loading Buffer II to each of the samples.

Note: There is no need to pre-heat DNA sample.

c. Load the DNA ladder and samples into the gel.

⚠ CRITICAL: Keep a space between samples and avoid the ends.

d. Run gel at 155 V for about 30 min.

Note: Until the purple dye touches the bottom of the gel.

e. While running, poke holes in 0.5 mL tubes using a 21 G needle and place inside 2 mL tubes.

Optional: you can use 0.5 mL Gel Breaker Tubes that already have holes, but you still need to increase the size of the holes by running a 21 G needle through each hole.

f. Stain the gel with ethidium bromide. Add 14 μL of 10 mg/mL EtBr to 200 mL of 1 × TBE buffer in a clean RNase-free tray. Add the gel and rock for 7 min.
g. Take a picture of the gel. Then cut the gel in the region corresponding to 168 bp to the end of signal using a clean razor blade and put the gel slice in the 0.5 mL tubes.

Note: Adapter-adapter dimer is 118 bp at this point, and the first RNA size selection is 50–250 nt, so the preferred size range in this selection is 168–368 bp.

⚠ CRITICAL: The razor blade needs to be sterilized with 100% EtOH and should be changed when cutting the gel lanes corresponding to each different sample to avoid sample cross-contamination.

h. Spin the samples at 14,000 rpm, 4°C for 2 min. Make sure most/all of the gel goes through the holes. If not, spin again for 1 min, poking more holes in the tubes if necessary.
i. Add 300 μL of 1 × NEB Buffer 2 and rotate for 2 h.
j. After 2 h, pipette the entire sample into a Spin-X column, and spin at 14000 rpm, 4°C for 2 min. Make sure all of the liquid spins out. There should be about 300 μL. Spin longer if necessary.
k. Precipitate the DNA by adding 3 μL of glycogen, 30 μL of 3M NaOAc (pH 5.5), and 900 μL of 100% EtOH.
l. Leave at −80°C at least 2 h.

Pause point: We usually leave the samples at −80°C overnight at this stage.

m. Centrifuge at maximum speed for at least 60 min at 4°C. If only at −80°C for 1 h, increase spin by 10 min.
n. Remove supernatant. Wash with 750 μL of 80% EtOH.
o. Centrifuge at maximum speed for 5 min at 4°C.
p. Pipette out as much of the supernatant as possible without disturbing the pellet. If not able to pipette it all out, allow the tubes to dry upright.
q. As soon as the pellet is dry, resuspend in 12 μL of Nuclease-free H_2O.
r. Quantify the library concentration using a Nanodrop and sequence the resulting libraries.

EXPECTED OUTCOMES

The bioanalyzer shows the sample with expected RNA fragment sizes and without contaminating adapter-adapter dimers (Figure 1).Troubleshooting 3.

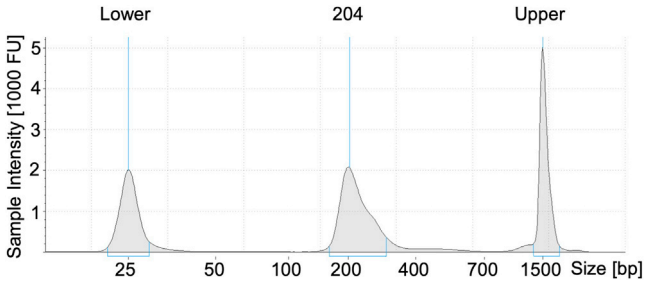

Figure 1. Example of a Bioanalyzer track for the final library from one NAD-seq samples with expected sizes from 168-368 bp, and no adapter-adapter dimers (missing peak at 118 bp)

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 1 day

The sequencing for at least 2 replicates of both NAD-seq and total RNA-seq produces at least 4 single-end fastq files for each genotype (Col-0 and *dxo1-2*). The raw data are trimmed to remove the adapter sequences using cutadapt (v1.9.1) with default parameters (Martin 2011). Trimmed reads are aligned to the Arabidopsis genome (TAIR10) using STAR (version 2.4.2a) (Dobin et al., 2013). HTSeq (v0.6.0) is used to calculate the raw reads mapping to given transcripts in a strand-specific manner (Anders et al., 2015). At least 1.5 GB RAM are required for running these command line codes for the Arabidopsis genome. Alternatively, Hisat2 (Kim et al., 2019) can be used for reads alignment.

Note: RAM requirements for STAR mapping: at least 10× bytes as compared to the genome size.

1. The command line commands for trimming adaptors

```
# For each fastq file in your experiment (*.fastq), run:
for file in *.fastq
do
cutadapt -a TGGAATTCTCGGGTGCCAAGGAACTCCAGTCACCCGTCCATCTCGTATGC -o $(echo $file |
sed 's/.fastq/_trimmed.fq/') $file > $(echo $file | sed 's/*.fastq/_cutadapt_report.txt/')
done
```

2. The command lines for building the genome index and mapping: Troubleshooting 5

```
STAR -runMode genomeGenerate -genomeDir TAIR10_star_index -genomeFastaFiles
TAIR10_genome.fasta
# For each file (*_trimmed.fq) following the adapter trimming step, run:
for file in *trimmed.fq
do
STAR -runThreadN 1 -genomeDir TAIR10_star_index -readFilesIn $file -outFilterMultimapNmax
10 -outFilterMismatchNmax 10 -outFilterMismatchNoverLmax 0.10 -outFileNamePrefix $(echo
$file | sed 's/_trimmed.fq//')
done
```

3. The command line code for counting raw read number in each detectable annotated transcript:

```
# For each alignment output file (*Aligned.out.sam), run:

for file in *Aligned.out.sam

do

htseq-count $file TAIR10_GFF3_genes.gff -f sam -q -t gene -i ID -m intersection-nonempty -

stranded=yes >$(echo $file | sed 's/Aligned.out.sam/_geneCount.txt/')

done
```

4. Finally, the NAD$^+$ capped RNA enrichment in NAD-seq compared to total RNA-seq is defined using DESeq2 (Love et al., 2014) with a False Discovery Rate (FDR) < 0.1. Alternatively, edgeR (McCarthy et al., 2012) can be used for defining NAD$^+$ capped RNA enrichment with the same statistical standard.

LIMITATIONS

Since the proportion of NAD$^+$ capped RNAs is relatively low among the total RNA population, the protocol for NAD-seq library construction requires 50–150 μg of total RNA as initial input, which may restrict its application from some small tissues such as embryos and pollen grains. The low RNA output after streptavidin bead pull down also increases the difficulty in adding a polyA$^+$ selection step to allow the specific detection of NAD$^+$ capped mRNAs.

TROUBLESHOOTING

Problem 1

At steps 5–7, low amount of RNAs are detected after Streptavidin bead pulldown.

Potential solution

Improve RNA input for ADPRC treatment followed by copper click chemistry, and make sure the ADPRC, azide-PEG3-biotin, and CuSO4 are freshly made.

Problem 2

At step 16, No DNA signals are observed in the third gel selection step after adaptor ligation, reverse transcription, and PCR amplification

Potential solution

Make sure the PNK treatment uses the ligase buffer that contains ATP, and 3′adaptor ligation uses the T4 RNA Ligase 2 truncated without addition of ATP, while 5′adaptor ligation uses T4 RNA Ligase 1 and includes the addition of ATP.

Problem 3

Step 19 shows that libraries contain a high abundance of adapter dimers.

Potential solution

Cut the gel carefully avoiding the adapter dimer size range in each step of gel-mediated size selection subsequent to adapter ligation.

Problem 4

At step 17, libraries contain a high abundance of ribosomal RNA (rRNA) reads once sequencing data is analyzed.

Potential solution

Optimize DSN treatment to ensure decrease of rRNA from the library preps.

Problem 5

At step 2 of Quantification and Statistical Analysis, unable to use the STAR tool for read alignment because of a large genome and not enough RAM.

Potential solution

Map reads to the genome using Hisat2 (Kim et al., 2019) instead, which requires less RAM as compared to the STAR tool.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Brian D. Gregory (bdgregor@sas.upenn.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The data sets analyzed during this study are available at GEO: GSE142390.

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AUTHOR CONTRIBUTIONS

B.D.G. conceived the study. X.Y., L.E.V. and B.D.G. designed and performed experiments and analyzed the data. X.Y. and B.D.G. wrote the paper with assistance from all authors. The authors have read and approved the manuscript for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq–a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166–169.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. *37*, 907–915.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Lyons, E., Pedersen, B., Kane, J., Alam, M., Ming, R., Tang, H., Wang, X., Bowers, J., Paterson, A., Lisch, D., et al. (2008). Finding and comparing syntenic regions among Arabidopsis and the outgroups papaya, poplar, and grape: CoGe with rosids. Plant Physiol. *148*, 1772–1781.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. *17*, 10.

McCarthy, D.J., Chen, Y., and Smyth, G.K. (2012). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. *40*, 4288–4297.

Winz, M.-L., Cahová, H., Nübel, G., Frindert, J., Höfer, K., and Jäschke, A. (2017). Capture and sequencing of NAD-capped RNA sequences with NAD captureSeq. Nat. Protoc. *12*, 122–149.

Yu, X., Willmann, M.R., Vandivier, L.E., Trefely, S., Kramer, M.C., Shapiro, J., Guo, R., Lyons, E., Snyder, N.W., and Gregory, B.D. (2021). Messenger RNA 5′ NAD+ capping is a dynamic regulatory epitranscriptome mark that is required for proper response to abscisic acid in Arabidopsis. Dev. Cell *56*, 125–140.