RNA Antisense Purification (RAP): Experimental Protocols

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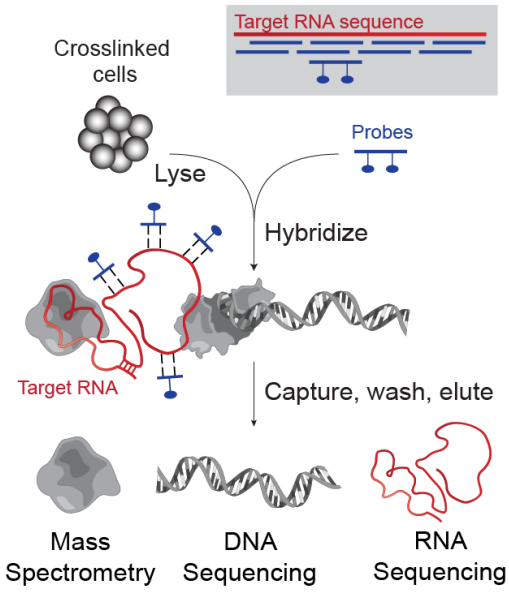

This document describes the experimental procedures for RNA Antisense Purification (RAP), a generally applicable method for biochemical purification of a specific RNA or RNAs. This technique can be used to enrich specific RNAs from clean, isolated RNA, or to purify endogenous RNA complexes from crosslinked cell extracts in order to identify interacting molecules.

In RAP, we crosslink cells to fix endogenous RNA complexes and then purify these complexes through hybrid capture with biotinylated antisense oligos. RNA or DNA molecules that interact with the target RNA are identified using high-throughput sequencing. RAP can be performed with various crosslinking conditions to identify RNA or DNA molecules that interact with the target RNA through different mechanisms; for instance, direct RNA-RNA interactions (via hybridization) may be specifically captured by crosslinking with psoralens, while indirect interactions (via protein intermediates) can be found by crosslinking with formaldehyde.

Compared to other similar techniques, the most distinctive and important feature of RAP is its use of long (>60-nucleotide) capture probes tiled across the entire target RNA. This probe design strategy robustly captures any RNA and enables the use of stringent hybridization and wash conditions that dramatically reduce nonspecific interactions of off-target nucleic acids or proteins.

The following document outlines the experimental protocols used in Engreitz *et al.* (2014) to purify various RNAs to examine their RNA and DNA interactions. For the latest RAP protocols, visit http://lncRNA.caltech.edu/RAP/. For additional information about RAP, see:

> Engreitz JM et al. "RNA-RNA Interactions Enable Specific Targeting of Noncoding RNAs to Nascent Pre-mRNAs and Chromatin Sites." *Cell*. September 2014.
>
> Engreitz JM et al. "The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome." *Science*. August 2013.

For questions and troubleshooting, email Jesse at engreitz@broadinstitute.org.

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1 Materials

1.1 Equipment

Sonication instrument (*e.g.*, Branson Sonifier with microtip) and chiller
Magnetic rack for 1.5 mL tubes (*e.g.*, Invitrogen DynaMag-2)
PCR machine and real-time quantitative PCR machine
Microcentrifuge
NanoDrop Spectrophotometer
Glass dounce
Heated mixer with 1.5 mL rack (*e.g.*, Eppendorf Thermomixer)
UV Stratalinker 2400 (for AMT crosslinking)
Agilent Bioanalyzer

1.2 Solutions

Different variants of the protocol require different combinations of the following solutions and buffers:

Scraping Buffer
1× PBS pH 7.5
0.5% BSA Fraction V
Store at 4°C

Cell Lysis Buffer
10 mM HEPES pH 7.5
20 mM KCl
1.5 mM $MgCl_2$
0.5 mM EDTA
1 mM tris(2-carboxyethyl)phosphine (TCEP, *add fresh)*
0.5 mM PMSF *(add fresh)*
Store at 4°C

Nuclear Lysis Buffer
20 mM HEPES pH 7.5
50 mM KCl
1.5 mM $MnCl_2$
1% NP-40
0.4% sodium deoxycholate
0.1% N-lauroylsarcosine
1 mM TCEP *(add fresh)*
0.5 mM PMSF *(add fresh)*
Store at 4°C

100× DNase Cofactor Solution
250 mM $MnCl_2$
50 mM $CaCl_2$

25× DNase Stop Solution
250 mM EDTA
125 mM EGTA

GuSCN Hybridization Buffer (1×)
20 mM Tris-HCl pH 7.5
7 mM EDTA
3 mM EGTA
150 mM LiCl
1% NP-40
0.2% N-lauroylsarcosine
0.1% sodium deoxycyolate
3 M guanidine thiocyanate
2.5 mM TCEP
Prepare 1× and 1.4x solutions

GuSCN Wash Buffer
20 mM Tris-HCl pH 7.5

10 mM EDTA
1% NP-40
0.2% N-lauroylsarcosine
0.1% sodium deoxycyolate
3 M guanidine thiocyanate
2.5 mM TCEP

RNase H Elution Buffer
50 mM Tris-HCl pH 7.5
75 mM NaCl
3 mM $MgCl_2$
0.125% N-lauroylsarcosine *(add fresh)*
0.025% sodium deoxycholate *(add fresh)*
2.5 mM TCEP *(add fresh)*

NLS Digestion Buffer
20 mM Tris-HCl pH 7.5
10 mM EDTA
2% N-lauroylsarcosine
2.5 mM TCEP

LiCl Hybridization Buffer (1×)
10 mM Tris-HCl pH 7.5
1 mM EDTA
500 mM LiCl
1% Triton X-100
0.2% SDS
0.1 sodium deoxycholate
4 M urea

Low Stringency Wash Buffer
1× SSPE
0.1% SDS
1% NP-40
4 M urea

High Stringency Wash Buffer
0.1× SSPE
0.1% SDS
1% NP-40
4 M urea

FNK Buffer (5×)
50 mM Tris-HCl pH 7.5
5 mM $MgCl_2$
0.6 mM $CaCl_2$
50 mM KCl
10 mM DTT
0.01% Triton X-100

1.3 Additional Materials and Reagents

Different variants of the protocol use different combinations of the following reagents:

Oligonucleotide library or biotinylated probes (see section on Probe Generation)
NEBNext High-Fidelity PCR Master Mix (NEB)
T7 RNA Polymerase and 10× Buffer (NEB)
Murine RNase Inhibitor (NEB)
100 mM ATP, CTP, GTP, UTP (NEB)
DNA purification kit: DNA Clean and Concentrator-5 (Zymo)
RNA purification kits: RNA Clean and Concentrator-5 (Zymo), RNeasy Mini (Qiagen)
Bioanalyzer: RNA 6000 Pico Kit, Small RNA Kit, High-Sensitivity DNA Kit (Agilent)
TURBO DNase (Invitrogen)
Multiple-temperature reverse-transcription reagents (*e.g.*, AffinityScript Reverse Transcriptase and Buffer from Agilent)
Cell scraper
Disuccinimidyl glutarate (Pierce)

16% Formaldehyde solution in 10-mL ampules (Pierce)
2.5 M Glycine solution
BSA Fraction V
Ambion RNase Cocktail (Life Technologies)
4'-Aminomethyltrioxalen hydrochloride (AMT, Sigma-Aldrich)
Ambion 10× RNA Fragmentation Reagent (Life Technologies)
MyONE Streptavidin C1 magnetic beads (Invitrogen)
MyONE SILANE magnetic beads (Invitrogen)
Buffer RLT (Qiagen)
TRIzol (Life Technologies)
100 mM dithiothreitol (DTT)
10× RT Random Primers (Applied Biosystems)
RT-qPCR reagents and PCR primers
NEBNext Ultra DNA Library Prep Kit and Multiplex Primers (NEB)
FastAP Thermosensitive Alkaline Phosphtase (Thermo Scientific)
T4 Polynucleotide Kinase (NEB)
T4 RNA Ligase 1 (30 U / μL, NEB)
ExoSAP-IT (Affymetrix)
Agencourt AMPure XP magnetic beads (Beckman Coulter)
Low-retention pipette tips

2 Assay Design

1. Choose a target RNA of interest.
2. Choose appropriate negative control target sequences. Suggested controls include: (i) a scrambled version of the target RNA that represents a GC-content-matched control that does not exist in the cell; (ii) other similar-sized and similar-abundance RNAs that should have different interaction partners than the target RNA.
3. Choose appropriate positive control targets to aid in optimizing lysis and purification conditions. As a positive control, we suggest purifying the highly abundant U1 snRNA and comparing the resulting data to that published in Engreitz et al. *Cell* 2014. In mouse cells (*Mus musculus*), use the following three 5'-biotinylated probes:

```
1. CAGGGGAGAGCGCGAACGCAGTCCCCCACTACCACAAATTATGCAGTCGA
2. GTTTCCCGCATTTGGGGAAATCGCAGGGGTCAGCACACCCCAAAGTGCAA
3. TGGGTGAGCCTCGCCCTGGGAAAACCACCTTCATGATCATGGTATCTCCC
```

3 Probe Design and Generation

Goal: Design and generate long antisense biotinylated ssDNA probes that tile across the length of the target RNA. The goal is to robustly capture the target RNA, regardless of secondary structure or RNA-protein interactions, while avoiding off-target hybridization and capture of other sequences.

The method described below uses oligonucleotide library synthesis strategies to simultaneously generate pools of oligos targeting many genes of interest. Each probe set is tagged with unique PCR primers that allow for enrichment of specific probe sets from the total pool. Probe sets are *in vitro* transcribed and then reverse transcribed with biotinylated primers to generate single-biotin ssDNA probes. An alternative strategy for obtaining RAP probes is to order 5' biotinylated oligos from a commercial supplier. Compared to the protocol presented below, ordering ready-to-use biotinylated probes from a commercial supplier potentially provides a faster and cheaper alternative for obtaining large amounts of a smaller number of probes.

RAP uses highly denaturing and stringent hybridization conditions to ensure capture specificity. However, nonspecific interactions are currently difficult to predict and will be different for each new target RNA, and so we recommend using two independent probe sets in an even/odd design to provide additional confidence in RNA-chromatin interactions identified with RAP.

We note that single-stranded DNA probes provide better capture specificity than the RNA probes used in previous iterations of this protocol.

3.1 Probe Design

1. Design 90- to 120-nucleotide capture probes antisense to the target RNA sequences. Shorter probes (50-90 nucleotides) can also be used, provided GC content is >50%. Probes can be tiled across the entire transcript (*e.g.*, each 120-nucleotide probe overlaps the next probe by 105 nucleotides), or they can be divided into two non-overlapping probe pools (even and odd) for additional specificity.
2. Omit probes that may hybridize to off-target sequences. Remove probes that contain more than 8 bases of any repetitive or low-complexity sequences as defined by RepeatMasker and Tandem Repeat Finder (these annotations can be viewed on the UCSC Genome Browser at http://genome.ucsc.edu). Remove probes that contain homo-polymers of more than 8 bases. Remove probes that align to other regions in the genome with 25 or more matching bases (*e.g.*, with BLAT).
3. Choose and validate RT-qPCR primers spanning a short amplicon (<90 bases) on the target RNA, and omit probes that overlap this region. This will enable qPCR measurement of purification yields and enrichments without confounding signal from residual amounts of the probes themselves.

4. For each probe set, design unique PCR tags (20 base-pairs) with 65 °C annealing temperatures (hereafter, Left Tag Primer and Right Tag Primer). Append the PCR tags to the ends of each probe in a given probe set such that the Left Tag Primer is on the 5' end of the oligo sequence and the Right Tag Primer is on the 3' end of the oligo sequence (which is itself antisense to the target RNA).
5. Order the pool of ssDNA oligos from an oligo library synthesis company (such as Agilent Technologies or CustomArray, Inc.). To improve synthesis of difficult sequences (such as probes containing poly-T sequences), synthesize both the desired ssDNA oligo as well as its reverse complement in the same pool.
6. Resuspend the lyophilized oligo pool in water to a final concentration of 10 nM.
7. Order enrichment primers: for each probe set, order (i) Left Tag and Right Tag primers; (ii) T7-Left Tag and T7-Right Tag Primers, which are comprised of Left Tag and Right Tag Primers each following a T7 promoter sequence (*e.g.*, GGATTCTAATACGACTCACTATAGGG-Left Tag Primer); and (iii) 5'-biotinylated Left Tag and Right Tag Primers.

3.2 Probe Set Enrichment PCR

8. For each probe set, enrich the component probes from the oligo pool using the unique PCR tags. Set up PCR reaction on ice:

Oligo Pool (10 nM)	1 µl
Tag Primer Left (25 µM)	1 µl
Tag Primer Right (25 µM)	1 µl
NEBNext High-Fidelity 2x Master Mix (NEB)	25 µl
H_2O	23 µl
Total	50 µl

9. Run PCR program to enrich the desired subset of oligos from the pool:

Initiation Denaturation	98°C	30 seconds	1 cycle
Denaturation	98°C	10 seconds	
Annealing	65°C	20 seconds	25-35 cycles
Extension	72°C	20 seconds	
Final Extension	72°C	60 seconds	1 cycle
Hold	4°C	hold	

10. Clean up the PCR product using the Qiagen PCR Purification Kit according to manufacturer's protocol. Measure DNA yield using a NanoDrop spectrophotometer. Examine the dsDNA product on an agarose gel. If necessary, repeat Step 2 varying the annealing temperature or number of cycles until the reaction produces a single clean band of the appropriate size.
11. Create a dilution of the enriched PCR product that is approximately 1 nM.
12. Perform a second round of amplification to add the T7 promoter sequence. If desired, set up two PCR reactions to add the T7 promoter sequence onto one or the other end of the dsDNA template, allowing for generation of both antisense and sense probes

during the *in vitro* transcription step. To generate antisense ssDNA probes that capture the target RNA, for example, use Tag Primer Left with T7-Tag Primer Right.

Diluted enriched dsDNA (~1 nM)	1 µl
Primer mix (25 µM of each primer)	2 µl
Phusion High-Fidelity 2× Master Mix (NEB)	25 µl
H_2O	23 µl
Total	50 µl

Initial Denaturation	98°C	30 seconds	1 cycle
Denaturation	98°C	10 seconds	
Annealing	60°C	20 seconds	3 cycles
Extension	72°C	20 seconds	
Denaturation	98°C	10 seconds	
Annealing	68°C	20 seconds	9-12 cycles
Extension	72°C	20 seconds	
Final Extension	72°C	60 seconds	1 cycle
Hold	4°C	hold	

13. Clean the PCR product using the Qiagen PCR Purification Kit according to manufacturer's protocol. Examine the dsDNA product on an agarose gel. Assess DNA yield using a NanoDrop spectrophotometer. If necessary, repeat PCR varying the annealing temperature or number of cycles until the reaction produces a single clean band of the appropriate size. If necessary, perform multiple PCR reactions to generate >250 ng of dsDNA T7 template for *in vitro* transcription.

3.3 *In Vitro* Transcription of Probe DNA Template

14. For each probe set, set up one or more 40 µL *in vitro* transcription reactions:

T7 DNA template (~250 ng)	24.2 µl
10× RNA Polymerase Reaction Buffer (NEB)	4 µl
100 mM ATP	2 µl
100 mM CTP	2 µl
100 mM GTP	2 µl
100 mM UTP	2 µl
T7 RNA Polymerase (NEB)	3 µl
100 mM DTT	0.4 µl
Murine RNase Inhibitor (NEB)	0.4 µl
Total	40 µl

15. Mix well by pipetting and incubate at 37°C overnight.
16. Next day, denature RNA/DNA hybrids by incubating at 85°C for 3 minutes. Afterward, place immediately on ice for 1 minute.

17. To digest dsDNA templates, add 42 μL H_2O, 10 μL TURBO DNase Buffer, and 8 μL TURBO DNase (100 μL total volume). Incubate at 37 °C for 15 minutes. Note: TURBO DNase outperforms DNase I at high salt concentrations.
18. Incubate at 37°C for 15 minutes to digest T7 DNA template.
19. Purify RNA with the RNeasy Mini kit (Qiagen) using sufficient ethanol to precipitate the ~150-nucleotide RNA fragments: Add 3.5× RLT (350 μL) to sample and mix well. Add 1.5× 100% ethanol (775 μL) to sample-RLT mixture and mix well. Transfer 700 μL to the RNeasy Mini column, and spin for 15 seconds at >8,000× *g*. Discard flow-through and repeat with remaining sample. Add 500 μL Buffer RPE to column and spin for 15 seconds. Discard flow-through. Repeat wash step. Transfer column to fresh collection tube and spin for 2 minutes to remove residual Buffer RPE. Transfer column to 1.5 mL tube; add 30 μL H_2O and spin for 1 minute to elute.
20. Measure RNA yield with a NanoDrop and dilute to a convenient concentration (*e.g.*, 1 μg/μL). Ideally yield should be >50 μg for a 40 μL reaction.
21. Denature RNA and run on gel to confirm the correct size of the *in vitro* transcription product.

3.4 Reverse Transcription to Generate ssDNA Probes

22. To generate ssDNA, set up a 200 μL reverse transcription reaction:

RNA template (10 μg) + H_2O	120	μl
100 µM 5'-biotinylated Left Tag Primer	20	μl
10× AffinityScript Buffer	20	μl
100 mM DTT	20	μl
100 mM dNTPs (25 mM each)	8	μl
AffinityScript Reverse Transcriptase Enzyme	10	μl
Total	200	μl

23. Incubate at 55°C for 50 minutes, then 75°C for 5 minutes.
24. To degrade RNA templates, add 0.1× (20 μL) 1 M NaOH. Incubate at 75 °C for another 10 minutes.
25. Add 0.1× (20 μL) 1 M acetic acid to neutralize.
26. Clean up the ssDNA product and eliminate the unused primer by size selection. For example, use a Zymo RNA Concentrator-5 column: Add 2× volume RNA Binding Buffer (480 μL) and mix well; add 1.9× original volume 100% ethanol (456 μL) and mix well. Transfer 700 μL to column; spin at 12,000× g for 1 minute. Discard flow-through and repeat with remaining sample. Add 400 μL RNA Prep Buffer; spin for 30 seconds and discard flow-through. Add 700 μL RNA Wash Buffer; spin and discard flow-through. Repeat the wash step with 400 μL RNA Wash Buffer. Transfer column to clean collection tube and spin for 2 minutes. Transfer column to a 1.5 mL tube, add 30 μL of water, and spin at 10,000× *g* for 1 minute to elute.

27. Measure the yield with a NanoDrop. Ideal yield should be ~3 μg ssDNA for a 200 μL reverse transcription reaction.
28. Freeze biotinylated ssDNA probe until use.

4 Magnetic-bead Purification of Nucleic Acids

Goal: Clean up RNA or DNA using magnetic beads.

4.1 SILANE

At several steps, we use SILANE beads to clean up samples in place of column purifications or chloroform extractions. Size selection can be achieved through adjusting the salt and alcohol concentrations during the initial binding. Use this protocol for a basic SILANE clean-up (captures all nucleic acids >20 nucleotides):

1. Aliquot out an appropriate volume of MyONE SILANE magnetic beads for the clean-up (capacity is at least 100 ng RNA and/or DNA per 5 μl of SILANE beads).
2. Place tube with SILANE beads on a magnetic rack. Wait ~30 seconds for beads to completely separate.
3. Remove supernatant, then remove tube from rack and resuspend beads once in Buffer RLT (Qiagen).
4. Place on magnet, wait for beads to separate, and remove supernatant.
5. Resuspend beads in 3.5× sample volume Buffer RLT (Qiagen) and add to sample (*e.g.*, for cleaning a 20 μL reaction, add beads in 70 μL of RLT).
6. Add 4.5× original sample volume 100% isopropanol (*e.g.*, for cleaning a 20 μL reaction, add 90 μL isopropanol) and mix well by pipet. *Note: In some SILANE bead purifications we use ethanol instead of isopropanol.*
7. Incubate at room temperature for 2 minutes.
8. Place tube on magnetic rack. Wait 1-2 minutes for beads to separate, then remove and discard supernatant.
9. Wash by removing tube from magnetic rack, resuspending beads in 70% ethanol (*e.g.*, 100-300 μL), capturing beads, and removing supernatant. Repeat wash step for a total of two washes.
10. Carefully remove all remaining 70% ethanol. Dry beads on the magnetic rack for 4-6 minutes or until dry (exact timing depends on bead volume, humidity, etc.).
11. Elute by resuspending beads in desired volume of H_2O, then magnetically separating and transferring eluate to a new tube.

4.2 SPRI

We use Agencourt AMPure XP (SPRI) beads to clean up and size-select dsDNA libraries. Use this protocol:

1. Add indicated volume SPRI beads to the DNA reaction. Take care when pipetting the viscous SPRI bead solution. Mix well by pipetting and wait 5 minutes.
2. Place on magnet and wait 2-5 minutes.

3. Remove and discard supernatant.
4. Wash beads twice in 100 μL 70% ethanol: add ethanol to beads while tube is on magnet, then move tube around magnet so that beads fly through ethanol from side to side.
5. Dry beads for 5-10 minutes.
6. Add H_2O to elute.

5 Sample Preparation

Goal: Crosslink cells to fix *in vivo* RNA complexes. Lyse cells and fragment RNA and/or DNA to appropriate sizes. Multiple protocols below describe sample preparation for different variants of the RAP protocol, including using AMT crosslinking (for identifying direct RNA-RNA interactions), formaldehyde crosslinking (for identifying direct and indirect RNA-RNA), or formaldehyde-DSG crosslinking (for identifying direct and indirect RNA-RNA and RNA-chromatin interactions).

Optimization of the lysis conditions (amount of sonication, amount/timing of DNase) is a critical step in establishing the protocol for the first time. The length of sonication might vary from 1-10 minutes and DNase treatment might vary from 10 to 20 minutes, depending on cell number, ploidy, crosslinking strength, and the desired RNA fragment size. To optimize DNase timing and conditions, remove 5 μL lysate aliquots every 2-4 minutes, quench with EDTA and EGTA on ice, and assay RNA and/or DNA sizes for each time point as described in the protocol. We recommend trying several different lysis conditions and comparing the results obtained when performing RAP on a positive control target RNA. If an appropriate combination of solubilization and RNA/DNA fragment sizes cannot be obtained by varying the amount of sonication or DNase, then reducing the strength of the crosslinking may be necessary.

These protocols describe the steps for adherent cells, but can be readily adapted for cells grown in suspension.

5.1 RAP-DNA and RAP-RNA with Formaldehyde-DSG Crosslinking

5.1.1 Cell Harvesting and Crosslinking

1. Grow adherent cells on 15-cm plates. Before crosslinking, carefully split and count one plate. Note: Accurate cell counts are critical for maintaining consistency between cell and lysate batches because cell numbers affect the efficiency of the sonication and DNase treatment during lysis. We typically harvest and crosslink 100-200 million cells in parallel and freeze multiple 20-million cell pellets; one of these pellets is spent to optimize lysis conditions, and the rest are used for purification experiments.
2. Heat one aliquot PBS at 37 °C and chill one aliquot at 4 °C (see below for volumes).
3. Resuspend 50-mg of DSG in 306 μl DMSO to create a 0.5 M stock solution of DSG.
4. Dilute DSG to 2 mM in PBS. Prepare 7 mL of 2 mM DSG for each 15-cm plate.
5. Remove media from cells. Rinse cells in plate with 10 mL room temperature PBS. Discard PBS.
6. Add 7-10 mL of 2 mM DSG solution and rock plates gently at room temperature for 45 minutes to crosslink.

7. Immediately before using, prepare a 3% formaldehyde solution in PBS preheated to 37 °C. Use a fresh ampule of 16% formaldehyde (Pierce).
8. Remove DSG solution from cells and wash once with room temperature PBS.
9. Add 7 mL warmed 3% formaldehyde solution to cells. Incubate at 37 °C for 10 minutes, gently rocking by hand every 3 minutes.
10. Quench formaldehyde crosslinking by adding glycine to a final concentration of 500 mM. Incubate at 37 °C for 5 minutes.
11. Discard formaldehyde waste in appropriate disposal container.
12. Rinse cells three times with cold PBS. Avoid dislodging cells from plate.
13. After last wash, add 2 mL of ice-cold Scraping Buffer to each 15-cm plate. From this point, keep cells at 4 °C.
14. Scrape cells from plate and transfer to a 15-mL Falcon tube.
15. Centrifuge at 1000× *g* at 4°C for 5 minutes to pellet cells.
16. Discard supernatant and resuspend pellet in 1 mL ice-cold Scraping Buffer to break up the pellet. Add more Scraping Buffer if necessary for convenient aliquoting (*e.g.*, add 1 mL of Scraping Buffer for every 20 million cells).
17. Aliquot cells into microcentrifuge tubes (20 million cells each) and spin at 2,000× *g* at 4 °C for 5 minutes.
18. Remove supernatant and flash freeze pellet in liquid nitrogen. Store until cell lysis at -80°C.

5.1.2 Cell Lysis

Note: All steps and buffers should be cooled to 4°C unless otherwise stated.

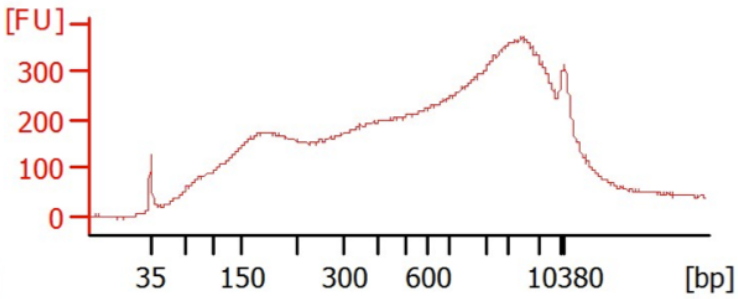
19. Thaw cell pellets by completely resuspending 20 million cells in 1 mL Hypotonic Cell Lysis Buffer (add TCEP and PMSF fresh) in a 1.5 mL microcentrifuge tube.
20. Pellet cells by spinning at 3300× *g* for 7 minutes. Remove supernatant.
21. Gently resuspend swelled cells in 1 mL ice cold Cell Lysis Buffer pre-mixed with 0.1% NP-40. Incubate on ice for 10 minutes.
22. Transfer to an ice-cold glass dounce of appropriate size (e.g., 2 mL). Homogenize cell lysate by douncing 20×.
23. Transfer cells back to a microcentrifuge tube and pellet nuclei by spinning at 3300× g for 7 minutes. Remove supernatant.
24. Resuspend nuclei in 1 mL of Nuclear Lysis Buffer (add TCEP and PMSF fresh). Incubate on ice for 10 minutes.
25. Sonicate using a Branson Sonifier fitted with a microtip using 5 Watts of power for 2 minutes in pulses (0.7 seconds on, 3.3 seconds off). Samples should be kept cold during sonication, for example by holding the sample in a 4 °C chilling rack or ice bath. Depending on the length of sonication and the efficiency of the cooling strategy,

longer breaks between sonication pulses may help to keep samples cool. Note again that this step is very sensitive to cell count, type of chilling rack, shape and size of tube, etc., and so plan to optimize by testing multiple conditions.

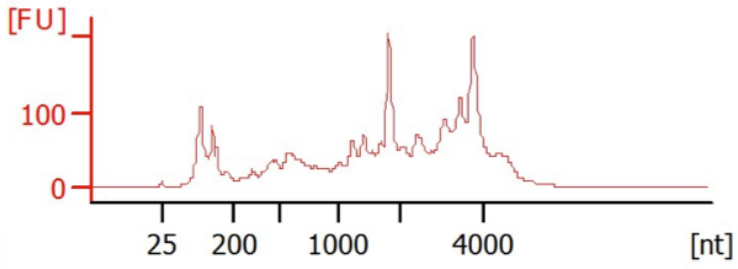
26. Split sample into two separate microcentrifuge tubes, each with 500 μL lysate.
27. To each, add 6 μL of 100× DNase Cofactor Solution and 30-100 μL TURBO DNase. Mix by pipet.
28. Transfer to a 37 °C heat block and incubate for 10-15 minutes.
29. Return sample to ice and immediately halt DNase reaction by adding 24 μL of DNase Stop Solution. Mix immediately by pipetting.
30. Remove and save a 5 μL aliquot of lysate (pre-clear).
31. Mix ~600 μL of lysate with 1.5 mL of 1.4× concentrated GuSCN Hybridization Buffer.
32. Clear lysate by spinning at maximum speed (16,000× *g*) for 10 minutes.
33. Remove and save a 5 μL aliquot of lysate (post-clear).
34. Flash-freeze aliquots of lysate in liquid nitrogen and store at -80 °C.

5.1.3 Check RNA and DNA Sizes

35. To check quantities and sizes of RNA and DNA in saved aliquots of lysate (Steps 12 and 15 above), first add 40 μL of NLS Digestion Buffer, 2.5 μL of 5 M NaCl, and 2.5 μL Proteinase K, and incubate at 65°C for 60 minutes.
36. Clean and purify nucleic acids using 10 μL of SILANE beads. At end, add 20 μL H_2O but do not remove from beads.
37. Split sample/bead mixture in half: treat half with 1 μL TURBO DNase + 1 μL TURBO DNase Buffer, and the other half with 1 μL of Ambion RNase Cocktail. Incubate each for 37 °C for 10 minutes.
38. Clean and purify nucleic acids using the SILANE beads already in the mixture. Elute in 10 μL H_2O.
39. Quantify RNA and DNA yields with a NanoDrop spectrophotometer. Accounting for the dilution of the lysate between the pre-clear and post-clear aliquots, we typically find that the post-clear aliquots contains >50% of the DNA of the pre-clear aliquot, indicating successful solubilization of chromatin.
40. Assess fragment sizes with the Agilent Bioanalyzer (High-Sensitivity DNA and RNA Pico kits). The figures below show ideal sizes, although some variation is expected and may not significantly change the efficiency of the purification.



DNA sizes after lysis
(High-Sensitivity DNA Bioanalyzer)



RNA sizes after lysis
(RNA 6000 Pico Bioanalyzer Assay)

5.2 RAP-RNA with Formaldehyde Crosslinking

We also use this protocol for non-crosslinked controls.

Note: Differences from the formaldehyde-DSG protocol above are printed in red text.

5.2.1 Cell Harvesting and Crosslinking

1. Grow adherent cells on 15-cm plates. Before crosslinking, carefully split and count one plate. Note: Accurate cell counts are critical for maintaining consistency between cell and lysate batches because cell numbers affect the efficiency of the sonication and DNase treatment during lysis. We typically harvest and crosslink 100-200 million cells in parallel and freeze multiple 20-million cell pellets; one of these pellets is spent to optimize lysis conditions, and the rest are used for purification experiments.
2. Heat one aliquot PBS at 37 °C and chill one aliquot at 4 °C (see below for volumes).
3. Remove media from cells. Rinse cells in plate with 10 mL room temperature PBS. Discard PBS.
4. Immediately before using, prepare a 2% formaldehyde solution in PBS preheated to 37 °C. Use a fresh ampule of 16% formaldehyde (Pierce).
5. Add 7 mL warmed 2% formaldehyde solution to cells. Incubate at 37 °C for 10 minutes, gently rocking by hand every 3 minutes.
6. Quench formaldehyde crosslinking by adding glycine to a final concentration of 500 mM. Incubate at 37 °C for 5 minutes.
7. Discard formaldehyde waste in appropriate disposal container.
8. Rinse cells three times with cold PBS. Avoid dislodging cells from plate.
9. After last wash, add 2 mL of ice-cold Scraping Buffer to each 15-cm plate. From this point, keep cells at 4 °C.
10. Scrape cells from plate and transfer to a 15-mL Falcon tube.
11. Centrifuge at 1000× *g* at 4°C for 5 minutes to pellet cells.
12. Discard supernatant and resuspend pellet in 1 mL ice-cold Scraping Buffer to break up the pellet. Add more Scraping Buffer if necessary for convenient aliquoting (*e.g.*, add 1 mL of Scraping Buffer for every 20 million cells).
13. Aliquot cells into microcentrifuge tubes (20 million cells each) and spin at 2,000× *g* at 4 °C for 5 minutes.
14. Remove supernatant and flash freeze pellet in liquid nitrogen. Store until cell lysis at -80°C.

5.2.2 Cell Lysis

Note: All steps and buffers should be cooled to 4°C unless otherwise stated.

15. Thaw cell pellets by completely resuspending 20 million cells in 1 mL Hypotonic Cell Lysis Buffer (add TCEP and PMSF fresh) in a 1.5 mL microcentrifuge tube.
16. Pellet cells by spinning at 3300× *g* for 7 minutes. Remove supernatant.
17. Gently resuspend swelled cells in 1 mL ice cold Cell Lysis Buffer pre-mixed with 0.1% NP-40. Incubate on ice for 10 minutes.
18. Transfer to an ice-cold glass dounce of appropriate size (e.g., 2 mL). Homogenize cell lysate by douncing 20×.
19. Transfer cells back to a microcentrifuge tube and pellet nuclei by spinning at 3300× g for 7 minutes. Remove supernatant.
20. Resuspend nuclei in 1 mL of GuSCN Hybridization Buffer.
21. Sonicate using a Branson Sonifier fitted with a microtip using 10 Watts of power for 7 minutes in pulses (0.7 seconds on, 3.3 seconds off). Samples should be kept cold during sonication, for example by holding the sample in a 4 °C chilling rack or ice bath. Depending on the length of sonication and the efficiency of the cooling strategy, longer breaks between sonication pulses may help to keep samples cool.
22. Add GuSCN Hybridization Buffer to bring total volume to 20 million cells in 4 mL.
23. Remove and save a 5 μL aliquot of lysate (pre-clear).
24. Clear lysate by spinning at maximum speed (16,000× *g*) for 10 minutes.
25. Remove and save a 5 μL aliquot of lysate (post-clear).
26. Flash-freeze aliquots of lysate in liquid nitrogen and store at -80 °C.
27. Check RNA and DNA fragment sizes as above.

5.3 RAP-RNA with Psoralen (AMT) Crosslinking

5.3.1 Cell Harvesting and Crosslinking

1. Grow adherent cells on 15-cm plates. Prepare plates for both crosslinked (+AMT) and mock-crosslinked (-AMT control) conditions.
2. Prepare 0.5 mg/mL AMT solution in PBS: Resuspend AMT in H_2O at 1 mg/mL and then add an equal volume of 2× PBS. Chill solution in the dark on ice.
3. Remove media from cells. Rinse cells in plate with 10 mL room temperature PBS. Discard PBS.
4. Trypsinize and pellet cells, then wash once more with PBS. Spin and discard supernatant.
5. Resuspend cell pellet (~25 million cells) in 4 mL of ice-cold AMT solution (+AMT) or ice-cold PBS alone (-AMT control). Incubate the cells on ice for 15 minutes.
6. Transfer samples to a pre-chilled 10-cm tissue culture dish.
7. Place cells on ice under a long-wave UV bulb (350 nm) in a UV Stratalinker 2400 (Stratagene). Cells should be approximately 3-4 cm away from the light source.
8. Expose cells to UV light at maximum power for 7 minutes. Mix every 2 minutes.
9. Transfer irradiated cells to cold tubes and spin at 330× *g* for 4 minutes. Discard supernatant.
10. Isolate crosslinked RNA using TRIzol reagent (Life Technologies) according to manufacturer's protocol. Clean RNA with Zymo RNA Concentrator-25 column.
11. Quantify nucleic acid yields with a NanoDrop spectrophotometer.
12. Digest DNA: For each 8 μg of purified nucleic acid, set up a 50 μL reaction (5 μL 10× TURBO DNase Buffer, 2.5 μL TURBO DNase). Incubate at 37°C for 20 minutes. Clean with RNA Concentrator-5 column (Zymo), and elute in 36 μL H_2O.
13. Measure RNA yield with a NanoDrop spectrophotometer.
14. Store at -80°C or proceed directly to fragmentation.

5.3.2 RNA Preparation and Fragmentation

15. Prepare 2 μg of input RNA per sample.
16. Fragment RNA to ~100 nucleotides: Start with 4 μg of RNA in 36 μL H_2O. Add 4 μL 10× RNA Fragmentation Reagent (Ambion). Heat sample for precisely 3 minutes at 70°C and then transfer immediately to ice.
17. Clean the reaction with Zymo RNA Concentrator-5 column and elute in 20 μL H_2O.
18. Store at -80°C or proceed directly to RAP-RNA$^{[AMT]}$.

6 RNA Antisense Purification

Goal: Purify target RNA with biotinylated probes. Perform the experiment in parallel for controls (*e.g.*, other RNAs or non-crosslinked lysate).

6.1 RAP-DNA and RAP-RNA with Formaldehyde or Formaldehyde-DSG Crosslinking

6.1.1 Lysate Preparation

1. Thaw lysate corresponding to 5 million cells for each sample (~1 mL).
2. Aliquot out 100 μl MyONE Streptavidin C1 magnetic beads (Invitrogen) for each purification from 5 million cells.
3. Wash beads twice in 0.5× original bead volume Hybridization Buffer, using a magnetic rack to capture beads and remove wash buffers each time. When separating magnetic beads from solution, place sample on magnet and wait 1-2 minutes before proceeding to allow beads to completely separate.
4. Resuspend beads in 0.25× bead volume Hybridization Buffer. Add beads to lysate (*i.e.*, 25 μL concentrated beads to 1 mL of lysate containing 5 million cells).
5. Incubate at 37 °C for 20-30 minutes, shaking.
6. Magnetically separate and transfer supernatant (streptavidin-cleared lysate) to a clean tube. Repeat this step to completely remove beads.
7. Save 5 μL of pre-cleared lysate (0.5% total input) on ice as DNA input.
8. Re-warm lysate to 37 °C, then proceed immediately to hybridization.

6.1.2 Hybridization, Capture, Wash

9. Prepare wash and elution buffers beforehand. Equilibrate solutions to the indicated temperatures before adding to samples.
10. Aliquot out 50 pmol of biotinylated ssDNA probe for each purification from 5 million cells.
11. Denature probe in H_2O at 85 °C for 3 minutes and then transfer immediately to ice.
12. Add probe to lysate, mix, and immediately transfer to a 37 °C Thermomixer.
13. Incubate at 37 °C for 2-3 hours, shaking at 1,200 r.p.m.
14. Just before use, aliquot out 500 μL of Streptavidin C1 magnetic beads for each sample. Wash twice in 0.5× bead-volume GuSCN Hybridization Buffer, then resuspend in 0.25× bead-volume GuSCN Hybridization Buffer. Add beads to sample and incubate at 37 °C for 15-30 minutes, shaking.
15. Magnetically separate and then remove supernatant. Optional: When establishing and troubleshooting the assay, it may be useful to save the supernatant, treat with Proteinase K, and isolate RNA and/or DNA to examine (i) the integrity of the RNA at the end of the hybridization, (ii) the amount of target RNA/DNA remaining in the

supernatant after capture, and (iii) the amount of probe remaining in the supernatant after capture.

16. Resuspend beads in 1× original bead-volume (500 μL) GuSCN Wash Buffer, then incubate at 45 °C for 3-10 minutes while washing other samples. Wash a total of 6 times. When removing final wash: place on magnet, remove liquid, spin down tube briefly, and remove the last drops of wash buffer with a fine tip.
17. Wash with 1× bead-volume RNase H Elution Buffer (add TCEP and detergents fresh).
18. Wash with 100 μL RNase H Elution Buffer (add TCEP and detergents fresh). Transfer samples to new tube before removing the final wash.
19. To elute, add 55 μL RNase Elution Buffer and 7.5 μL RNase H to each sample.
20. Incubate at 37 °C for 30 minutes, shaking.
21. Remove and save eluate.
22. Add 62.5 μL GuSCN Hybridization Buffer and incubate at 37 °C for 5 minutes, shaking.
23. Remove eluate and combine with previous eluate.
24. Magnetically separate the combined eluates once more and transfer to a new tube to remove any residual beads and attached ssDNA probe.
25. Add 312.5 NLS Digestion Buffer, 50 μL 5 M NaCl, and 12.5 μL Proteinase K to each sample and to the saved input sample. Proceed to DNA or RNA analysis section. Alternatively, divide sample for both RNA and DNA analysis.

6.1.3 RNA Elution and Analysis

26. Mix well, and incubate tubes for 1 hour at 65 °C to digest protein and reverse formaldehyde crosslinks.
27. Clean and purify nucleic acids using SILANE beads: To each sample, add 40 μL SILANE beads rinsed and resuspended in 50 μL 5 M NaCl. Mix well. Add 1× 100% isopropanol (550 μL). Mix well and wait 2 minutes. Place on magnet, and wash twice in 600 μL 70% ethanol. Dry for 10 minutes. Add 25 μL of H_2O and resuspend beads, but do not remove from beads. Transfer to a PCR strip tube, and clean once more by adding 87.5 μL RLT and 112.5 μL isopropanol. Wash twice in 70% ethanol and elute in 25 μL.
28. Proceed to quantitative real-time PCR (first digest residual genomic DNA and ssDNA probes by treating with DNase I and Exonuclease I) or RNA sequencing (see below). When setting up the assay, we recommend measuring the captured RNA using quantitative real-time PCR to determine enrichment and yield of the target RNA. Primers should include one or more primer pairs for the target RNA as well as multiple primer pairs targeting other abundant RNAs (*e.g.*, 18S rRNA, U1 snRNA).
29. RNA enrichments are typically in the range of 100-1,000-fold versus negative controls. RNA yields are typically in the range of 10-80%, depending on the target RNA. If low RNA (or DNA) enrichments are observed with high yield, one possible

reason is that crosslinked macromolecular complexes are too large due to over-crosslinking or insufficient sonication; try decreasing crosslinking or increasing sonication. If low RNA (or DNA) enrichments are observed with low yield, then there are multiple possibilities to consider. First, RNA may be degraded throughout the process, leading to poor capture; to address this, examine RNA integrity and yields at each intermediate step by RT-qPCR and/or visualization of RNA sizes with the Bioanalyzer. Second, the probe set may not properly capture the target RNA even with acceptable RNA integrity; to test this, use the same probe set to capture the target RNA in purified total RNA using the same protocol, and/or test the protocol in lysate using an abundant positive control RNA.

30. Calculate enrichment as the ratio of the amount of the target RNA in the target purification versus negative-control purification, normalized to the ratio of the signal of abundant RNAs in the target purification versus negative-control purification. Calculate yield as the ratio of the amount of the target RNA in the target purification versus the input, accounting for the fraction of input saved during the lysate preparation.

6.1.4 DNA Elution and Analysis

31. Mix well, and incubate tubes overnight at 60 °C to digest protein and completely reverse formaldehyde crosslinks.
32. Place samples on ice.
33. Clean and purify nucleic acids using SILANE beads: To each sample, add 40 μL SILANE beads rinsed and resuspended in 50 μL 5 M NaCl. Mix well. Add 1× 100% isopropanol (550 μL). Mix well and wait 2 minutes. Place on magnet, and wash twice in 600 μL 70% ethanol. Dry for 10 minutes. Add 25 μL of H_2O and resuspend beads, but do not remove from beads. Transfer to a PCR strip tube, and clean once more by adding 87.5 μL RLT and 112.5 μL isopropanol. Wash twice in 70% ethanol and elute in 25 μL.
34. In initial experiments, measure DNA yields and enrichments using quantitative PCR to validate that the experiment worked before moving immediately to DNA sequencing. Primers should include one or more primer pairs that measure genomic DNA close to but not overlapping the target gene locus; these regions should be strongly enriched (>100-fold) compared to input after normalizing to other locations in the genome. An appropriate negative control for this assay is comparing RAP with antisense probes to RAP with sense probes, which will capture DNA at the target locus but not RNA; the antisense probes should enrich more strongly for genomic DNA close to the target gene locus. Depending on the abundance of the target RNA, it may be necessary to use the entire DNA sample for qPCR, rather than saving some for DNA sequencing, to ensure that the DNA levels are high enough to meet the threshold for qPCR quantification.
35. Generate DNA sequencing libraries for high-throughput DNA sequencing (see below). For input sample(s), use 1/10 of the sample to avoid overloading the low-quantity DNA library preparation reaction.

36. Sequence the DNA libraries to generate ~20 million reads for the RAP samples and 30-80 million reads for the input samples. Proper analysis of the data, including identification of RNA-chromatin interaction sites and calculation of enrichments across different regions of the genome, requires deep sequencing of the input library because DNA fragment density can vary substantially across the genome.
37. For suggestions regarding RAP-DNA interpretation and analysis, please refer to Engreitz *et al*. Science 2013 and Engreitz *et al.* Cell 2014, particularly regarding the appropriate controls to ensure that observed peaks are not due to off-target hybridization.

6.2 RAP-RNA with AMT Crosslinking or RAP-RNA from Purified Total RNA

6.2.1 Hybridization, Capture, Wash, Elution

1. Prepare wash and elution buffers beforehand. Equilibrate solutions to the indicated temperatures before adding to samples.
2. Aliquot out 15 pmol of biotinylated ssDNA probe for each purification from 2 μg of input RNA.
3. Denature probe in H_2O at 85 °C for 3 minutes and then transfer immediately to ice.
4. Mix probe and input RNA in 300 μL of preheated LiCl Hybridization Buffer and immediately transfer to a 55°C Thermomixer.
5. Incubate at 55°C for 2 hours, shaking at 1,200 r.p.m.
6. Just before use, aliquot out 200 μL of Streptavidin C1 magnetic beads for each sample. Wash twice in 0.5× bead-volume LiCl Hybridization Buffer, then resuspend in 0.25× bead-volume LiCl Hybridization Buffer. Add beads to sample and incubate at 37 °C for 15-30 minutes, shaking.
7. Magnetically separate and then remove supernatant. Optional: When establishing and troubleshooting the assay, it may be useful to save the supernatant and isolate RNA to examine (i) the integrity of the RNA at the end of the hybridization, (ii) the amount of target RNA remaining in the supernatant after capture, and (iii) the amount of probe remaining in the supernatant after capture.
8. Wash three times in Low Stringency Wash Buffer: Resuspend beads in 1.25× original bead-volume (250 μL) wash buffer, then incubate at 58°C for 3-10 minutes while washing other samples.
9. Wash three times in 1.25× original bead-volume (250 μL) High Stringency Wash Buffer at 58°C. When removing final wash: place on magnet, remove liquid, spin down tube briefly, and remove the last drops of wash buffer with a fine tip.
10. Wash with 1× bead-volume RNase H Elution Buffer (add TCEP and detergents fresh).
11. Wash with 100 μL RNase H Elution Buffer (add TCEP and detergents fresh). Transfer samples to new tube before removing the final wash.
12. To elute, add 21 μL RNase Elution Buffer and 4 μL RNase H to each sample.
13. Incubate at 37 °C for 30 minutes, shaking.
14. Remove and save eluate.
15. Add 25 μL LiCl Hybridization Buffer and incubate at 37 °C for 5 minutes, shaking.
16. Remove eluate and combine with previous eluate.
17. Magnetically separate the combined eluates once more and transfer to a new tube to remove any residual beads and attached ssDNA probe. Place on ice.
18. Clean RNA with SILANE beads: Add 15 μL of beads resuspended in 150 μL RLT. Add 200 μL 100% ethanol. Bind for 2 minutes and remove supernatant. Wash twice

in 200 μL 70% ethanol. Dry beads for 6-10 minutes. Elute in 15 μL H_2O and remove eluate from beads.

19. Proceed to RNA sequencing.

7 RAP-DNA Sequencing Library Preparation

Goal: Prepare Illumina sequencing libraries from co-purified DNA. This protocol uses the NEBNext Ultra Library Prep Kit for Illumina and the NEBNext Multiplex Oligos for Illumina, with modifications we designed for making libraries from small amounts of short-fragment DNA.

1. Start with 12.5 μL of purified DNA in H_2O in low-retention PCR strip tubes.
2. Add 2.5 μL of master mix containing 1.5 μL 10× NEBNext End Repair Reaction Buffer and 1 μL NEBNext End Prep Enzyme Mix.
3. Mix by pipet and incubate at 20°C for 30 minutes.
4. Add 1 μL of 160 mM NaCl (15 mM final concentration) and mix.
5. Incubate at 55°C for 30 minutes, then hold at 4°C.
6. To the end repair reaction, add 1 μL of a 1:10 dilution of NEBNext Adaptor for Illumina. Then add 4 μL of master mix containing 3.75 μL of Blunt/TA Ligase Master Mix and 0.25 μL NEBNext Ligation Enhancer.
7. Mix and incubate at 20°C for 45 minutes.
8. Add 1 μL of USER enzyme. Mix and incubate at 37°C for 15 minutes.
9. Add 19 μL H_2O to 40 μL total volume.
10. Clean once using 0.7× volume (28 μL) SPRI beads. At end, add 40 μL H_2O but do not remove from beads.
11. Clean again use 1× volume (40 μL) SPRI beads. At end, elute in 24 μL and remove 23 μL from the beads.
12. Set up PCR reaction:

DNA	23	μl
NEBNext Indexed PCR Primer (25 μM)	1	μl
NEBNext Unversal PCR Primer (25 μM)	1	μl
NEBNext High-Fidelity 2× Master Mix (NEB)	25	μl
Total	50	μl

13. Run the following PCR program:

Initial Denaturation	98°C	30 seconds	1 cycle
Denaturation	98°C	10 seconds	
Annealing	67°C	30 seconds	4 cycles
Extension	72°C	30 seconds	
Denaturation	98°C	10 seconds	4-10* cycles
Annealing and Extension	72°C	30 seconds	
Final Extension	72°C	60 seconds	1 cycle
Hold	4°C	hold	

*RAP samples usually require 10 cycles during this step, while inputs require between 4 and 6.

14. Clean once using 1× volume (50 μL) SPRI beads. At end, add 50 μL H_2O but do not remove from beads.
15. Clean again use 1× volume (50 μL) SPRI beads. At end, elute in 13 μL H_2O.
16. Measure library concentration with Qubit fluorometric quantitation.
17. Examine DNA fragment sizes using the High-Sensitivity DNA Bioanalyzer kit.
18. Pool multiple barcoded libraries and sequence with Illumina.

8 RAP-RNA Sequencing Library Preparation

Goal: Prepare Illumina sequencing libraries from purified RNAs. This protocol is optimized for very low amounts of input RNA, and uses an adapter-ligation strategy in order to map locations of crosslinks (*e.g.*, for the AMT protocol). This RNA-sequencing protocol also includes several steps that remove contaminating ssDNA probes.

8.1.1 Primer and Adapter Sequences

RiL-19 RNA adapter: /5Phos/rArGrArUrCrGrGrArArGrArGrCrGrUrCrGrUrG/3ddC/
3Tr3 DNA adapter: /5Phos/AGATCGGAAGAGCACACGTCTG/3ddC/
5Phos = 5' phosphate, 3ddC = 3' dideoxycytosine to block self-ligation

AR17 primer for reverse transcription: ACACGACGCTCTTCCGA

Universal PCR primer:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

Barcoded PCR primer (example 8-mer barcode in red – Illumina indexing read will contain the reverse complement of this sequence):
CAAGCAGAAGACGGCATACGAGATCCTGGTAGGTGACTGGAGTTCAGACGTGTGCT
CTTCCGATCT

8.1.2 DNase Digestion and RNA Preparation

19. Start with 31 μL of purified RNA in H_2O in low-retention PCR strip tubes.
20. Add 19 μL of a master mix containing:

5× FNK Buffer	10	μl
Murine RNase Inhibitor	1	μl
FastAP Thermosensitive Alkaline Phosphatase	3	μl
T4 Polynucleotide Kinase	3	μl
TURBO DNase	1	μl
Exonuclease I	1	μl
Total	19	μl

21. Mix well and incubate at 37°C for 30 minutes.
22. Clean up with 15 μL SILANE beads, 3× volume RLT, and 1× volume ethanol. Elute in 6 μL H_2O.

8.1.3 First Ligation

23. Add 20 pmol (0.5 μL of 40 μM) RNA adapter RiL-19 to each sample.
24. Denature at 70°C for 2 minutes and then transfer immediately to ice.
25. Add 13.6 μL of ligation master mix:

10× NEB Ligase 1 Buffer	2	μl

DMSO (100%)	1.8	µl
ATP (100 mM)	0.2	µl
PEG 8000 (50%)	8	µl
Murine RNase Inhibitor	0.3	µl
T4 RNA Ligase 1 (30 U / µL)	1.3	µl
Total	13.6	µl

26. Close cap and mix by shaking vigorously and spinning down tubes many times.
27. Incubate at room temperature for 1.5 hours.
28. Clean RNA: Add 15 µL SILANE beads in 61 µL RLT. Mix well. Add 65 µL 100% ethanol. Mix well and wait 2 minutes. Wash twice by resuspending beads in 70% ethanol. Dry for 5-10 minutes. Elute in 14.3 µL H_2O.

8.1.4 Reverse Transcription – First Strand cDNA

1. To each sample, add 10 pmol (0.5 µL of 20 µM stock) AR17 primer.
2. Denature at 70°C for 2 minutes and then immediately transfer to ice.
3. Add 6 µL of reverse transcription master mix:

10× AffinityScript RT Buffer	2	µl
100 mM DTT	2	µl
100 mM dNTPs (25 mM each)	0.8	µl
Murine RNase Inhibitor	0.4	µl
AffinityScript Reverse Transcriptase Enzyme	0.8	µl
Total	6	µl

4. Quickly mix by pipet and transfer to a preheated 55°C incubator.
5. Incubate at 55°C for 5 minutes, 54°C for 50 minutes, then 4°C for 1 minute.
6. Immediately remove samples from thermal cycler.
7. Digest excess RT primers by adding 3 µL of ExoSAP-IT. Mix and incubate at 37°C for 12 minutes.

8.1.5 Probe Removal

8. Take 20 µL of Streptavidin C1 magnetic beads per sample. Wash in 1× C1 Binding Buffer (10 mM Tris pH 7.5, 250 mM LiCl, 20 mM EDTA, 0.1% Triton X-100). Aliquot out beads into an empty strip tube, place on magnet, then remove supernatant.
9. Add 2.5 µL of 10× C1 Binding Buffer to each sample, then transfer the samples to the washed beads and mix.
10. Incubate at 60°C for 15 minutes in a thermal mixer, shaking at 1,200 r.p.m.
11. Place samples on magnet; remove and keep supernatant. Discard beads with captured probes.

8.1.6 Second Ligation

12. Degrade RNA by adding 2.55 μL 1 M NaOH to each sample (100 mM final concentration).
13. Incubate at 70°C for 10 minutes.
14. Place samples on ice and neutralize solution by adding 2.55 μL 1 M acetic acid.
15. Clean up RNA using 12 μL of SILANE beads with 75 μL RLT and 65 μL 100% ethanol. At end, resuspend beads in 5.5 μL H_2O but do not remove sample from beads.
16. Add 40 pmol (0.5 μL of 80 μM stock) 3Tr3 DNA adapter to each sample containing cDNA and SILANE beads.
17. Denature at 75°C for 2 minutes and then transfer immediately to ice.
18. Add 14.1 μL of ligation master mix:

10× NEB Ligase 1 Buffer	2	μl
DMSO (100%)	0.8	μl
ATP (100 mM)	0.2	μl
PEG 8000 (50%)	9.5	μl
T4 RNA Ligase 1 (30 U / μL)	1.6	μl
Total	14.1	μl

19. Close cap and mix by shaking vigorously and spinning down tubes many times.
20. Incubate at room temperature overnight. Mix by shaking several times during this period.
21. Clean up sample by adding 5 μL more of SILANE beads in 61 μL RLT, then adding 55 μL 100% ethanol. Wash twice and elute in 25 μL H_2O.

8.1.7 PCR Enrichment

22. Set up PCR reaction:

cDNA	21	μl
Barcoded PCR primer (25 μM)	2	μl
Universal PCR primer (25 μM)	2	μl
NEBNext High-Fidelity 2× Master Mix (NEB)	25	μl
Total	50	μl

23. Run the following PCR program:

Initial Denaturation	98°C	30 seconds	1 cycle
Denaturation	98°C	10 seconds	
Annealing	67°C	30 seconds	4 cycles
Extension	72°C	30 seconds	
Denaturation	98°C	10 seconds	4-10* cycles
Annealing and Extension	72°C	30 seconds	
Final Extension	72°C	60 seconds	1 cycle

Hold	4°C	hold

*RAP samples usually require 10 cycles during this step, while inputs require between 4 and 6.

24. Clean once using 1.2× volume (60 μL) SPRI beads. At end, add 40 μL H_2O but do not remove from beads.
25. Clean again use 1.1× volume (50 μL) SPRI beads. At end, elute in 24 μL and remove 23 μL from the beads.
26. Measure library concentration with Qubit fluorometric quantitation.
27. Examine DNA fragment sizes using the High-Sensitivity DNA Bioanalyzer kit.
28. Pool multiple barcoded libraries and sequence with Illumina.