Chapter 11

RNA Antisense Purification (RAP) for Mapping RNA Interactions with Chromatin

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Abstract

RNA-centric biochemical purification is a general approach for studying the functions and mechanisms of noncoding RNAs. Here, we describe the experimental procedures for RNA antisense purification (RAP), a method for selective purification of endogenous RNA complexes from cell extracts that enables mapping of RNA interactions with chromatin. In RAP, the user cross-links cells to fix endogenous RNA complexes and purifies these complexes through hybrid capture with biotinylated antisense oligos. DNA loci that interact with the target RNA are identified using high-throughput DNA sequencing.

Key words RNA purification, Chromatin, Localization, lncRNA, Hybrid capture

1 Introduction

Large noncoding RNAs (lncRNAs)—a class of RNAs >200 nucleotides long that do not encode proteins—are emerging as critical regulators of gene expression in mammals [1–5]. Inspired in part by classical examples such as the X-inactive-specific transcript (Xist), a popular hypothesis is that many lncRNAs regulate gene expression by interacting with chromatin-regulatory proteins and recruiting them to specific DNA target sites. Indeed, recent work indicates that lncRNAs can interact with diverse chromatin regulators [6–9], can localize to specific sites in the genome [10–12], and may even establish nuclear subdomains that contain co-regulated DNA loci [12, 13]. Together, these observations highlight the need to map RNA-chromatin interactions to identify direct targets of lncRNA complexes.

Advances in RNA-centric biochemical purification have presented new opportunities for systematically mapping RNA interactions with chromatin [10–12]. RNA antisense purification (RAP) [12], as well as other recently developed methods like ChIRP [10] and CHART [11], captures a target RNA of interest through hybridization with antisense biotinylated oligos. By cross-linking

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endogenous macromolecular complexes prior to RNA capture, RAP allows for identification of proteins, RNA, and DNA loci that cross-link to and co-purify with the target RNA. Compared with previous approaches for examining RNA-chromatin interactions such as fluorescence in situ hybridization (FISH), RNA-centric biochemical purification enables genome-wide mapping of RNA-DNA interactions by coupling the protocol with high-throughput DNA sequencing (RAP-DNA). Thus, RAP provides an important tool for systematic interrogation of lncRNA function and mechanism.

Here, we describe the RAP technique for mapping RNA-chromatin interactions. Compared to similar protocols, the most distinctive and important feature of RAP is its use of long (120-nucleotide) capture probes tiled across the entire target RNA. This probe design strategy robustly captures any lncRNA and enables the use of stringent hybridization and wash conditions that dramatically reduce nonspecific interactions of off-target nucleic acids or proteins [12]. We have applied this protocol to investigate ncRNAs in mouse embryonic stem cells and fibroblasts; the method is readily adapted to other systems where large quantities of cells can be obtained.

2 Materials

Prepare all buffers and solutions with nuclease-free water. Follow appropriate regulations when handling chemicals (e.g., guanidine thiocyanate) and disposing waste.

2.1 Equipment

1. Sonication instrument (e.g., Branson Sonifier with microtip) and chiller.
2. Magnetic rack for 1.5 mL tubes (e.g., Invitrogen DynaMag-2).
3. PCR machine and real-time quantitative PCR machine.
4. Microcentrifuge.
5. NanoDrop spectrophotometer.
6. Glass dounce.
7. Heated mixer with 1.5 mL rack (e.g., Eppendorf ThermoMixer).

2.2 Solutions

Make stock solutions and store up to 6 months at room temperature, unless otherwise noted.

1. Scraping buffer: PBS and 0.5 % BSA Fraction V. Store at 4 °C.
2. Cell lysis buffer: 10 mM HEPES pH 7.5, 20 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM EDTA. Before use, add fresh 1 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Store at 4 °C.
3. Nuclear lysis buffer: 20 mM HEPES pH 7.5, 50 mM KCl, 1.5 mM $MnCl_2$, 1 % IGEPAL CA630 (NP-40), 0.4 % sodium

deoxycholate, and 0.1 % *N*-lauroylsarcosine. Before use, add fresh 1 mM TCEP and 0.5 mM PMSF. Store at 4 °C.

4. 100× DNase cofactor solution: 250 mM $MnCl_2$ and 50 mM $CaCl_2$.
5. 25× DNase stop solution: 250 mM EDTA and 125 mM EGTA.
6. 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 deoxycholate, 3 M guanidine thiocyanate, and 2.5 mM TCEP. Prepare both 1× and 1.4× stock solutions.
7. Wash buffer: 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 % NP-40, 0.2 % *N*-lauroylsarcosine, 0.1 % sodium deoxycholate, 3 M guanidine thiocyanate, and 2.5 mM TCEP.
8. RNase H elution buffer: 50 mM Tris-HCl pH 7.5, 75 mM NaCl, and 3 mM $MgCl_2$. Before use, add fresh 0.125 % *N*-lauroylsarcosine, 0.025 % sodium deoxycholate, and 2.5 mM TCEP.
9. NLS digestion buffer: 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 2 % *N*-lauroylsarcosine, and 2.5 mM TCEP.

2.3 Additional Materials and Reagents

1. NEBNext High-Fidelity PCR Master Mix (NEB).
2. T7 RNA polymerase and 10× buffer (NEB).
3. Murine RNase inhibitor (NEB).
4. 100 mM ATP, CTP, GTP, and UTP.
5. DNA purification kit: DNA Clean and Concentrator-5 (Zymo).
6. RNA purification kits: RNA Clean and Concentrator-5 (Zymo) and RNeasy Mini (Qiagen).
7. TURBO DNase (Invitrogen).
8. RNase H (5 U/μL, NEB).
9. Multiple-temperature reverse transcription reagents (e.g., AffinityScript reverse transcriptase and buffer from Agilent).
10. Cell scraper.
11. Disuccinimidylglutarate (Pierce).
12. 16 % formaldehyde solution in 10-mL ampules (Pierce).
13. 2.5 M glycine solution.
14. BSA Fraction V.
15. MyONE Streptavidin C1 magnetic beads (Invitrogen).
16. MyONE SILANE magnetic beads (Invitrogen).
17. 100 mM dithiothreitol (DTT).
18. 10× RT random primers (Applied Biosystems).
19. RT-qPCR reagents and PCR primers.
20. NEBNext Ultra DNA Library Prep Kit and Multiplex Primers (NEB).

3 Methods

3.1 Assay Design

1. Choose a target RNA of interest.
2. Choose appropriate negative control target sequences. For example, scramble the sequence of the target RNA to create a GC-content-matched control that does not exist in the cell. Alternatively, purify mRNAs, which likely do not interact with chromatin outside of their own genomic loci.
3. Choose appropriate positive control targets to aid in optimizing lysis and purification conditions (*see* **Note 1**).

3.2 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 nonoverlapping probe pools (even and odd) for additional specificity (*see* **Note 2**).
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 homopolymers 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.

3.3 Probe Generation

The protocol below uses oligonucleotide library synthesis technology to simultaneously generate pools of ssDNA probes targeting many different RNAs. 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 (*see* **Note 3**). For alternative probe synthesis and amplification strategies, *see* **Note 4**.

1. 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).

2. Order the pool of ssDNA oligos from an oligo library synthesis company (e.g., CustomArray, Inc.).
3. Resuspend the lyophilized oligo pool in water to a final concentration of 10 nM. Aliquot and store at–20 °C.
4. Order primers: for each probe set, order (1) Left Tag and Right Tag Primers; (2) 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., GGAT TCTAATACGACTCACTATAGGG-Left Tag Primer); and (3) 5′-biotinylated Left Tag and Right Tag Primers.
5. For each probe set, enrich the component probes from the oligo pool using the unique PCR tags. Set up PCR reaction on ice:
   - 1 μL of oligo pool (10 nM).
   - 1 μL of Tag Primer Left (25 μM).
   - 1 μL of Tag Primer Right (25 μM).
   - 25 μL of NEBNext High-Fidelity 2× Master Mix (NEB).
   - 22 μL H_2O.
6. Run PCR program:
   - 98 °C for 30 s.
   - 25–35 cycles of 98 °C for 10 s, 65 °C for 20 s, and 72 °C for 20 s.
   - 72 °C for 60 s.
   - 4 °C hold.
7. Clean the PCR product using a DNA purification column according to manufacturer's protocol. Measure DNA yield using a NanoDrop spectrophotometer. Examine the dsDNA product on an agarose gel. If necessary, repeat PCR varying the annealing temperature, oligo pool amount, or number of cycles until the reaction produces a single clean band at the appropriate size (*see* **Note 5**).
8. For each probe set, perform a second round of PCR amplification to add the T7 promoter sequence (GGATTCTAATAC GACTCACTATAGGG). 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 sense and antisense probes. To generate antisense ssDNA probes that capture the target RNA, for example, use Tag Primer Left with T7-Tag Primer Right.
   - 1 μL of diluted enriched dsDNA (~1 nM).
   - 2 μL of primer mix (mix contains 25 μM of each primer).
   - 25 μL of NEBNext High-Fidelity 2× Master Mix.
   - 22 μL H_2O.
9. Run PCR program:

- 98 °C for 30 s.
- 12–15 cycles of 98 °C for 10 s, 65 °C for 20 s, and 72 °C for 20 s.
- 72 °C for 60 s.
- 4 °C hold.

10. Clean the PCR product using a DNA purification column according to manufacturer's protocol. Measure DNA yield using a NanoDrop spectrophotometer. Examine the dsDNA product on an agarose gel. If necessary, repeat PCR varying the annealing temperature or number of cycles until the reaction produces a single clean band at the appropriate size. If necessary, perform multiple PCR reactions to generate >250 ng of dsDNA T7 template for in vitro transcription.
11. For each probe set, set up a 40 μL in vitro transcription reaction:
    - 24.2 μL T7 DNA template (250 ng total).
    - 4 μL 10× RNA polymerase reaction buffer.
    - 2 μL each 100 mM ATP, CTP, GTP, and UTP.
    - 3 μL T7 RNA polymerase.
    - 0.4 μL 100 mM DTT.
    - 0.4 μL murine RNase inhibitor.
12. Mix well by pipetting. Incubate at 37 °C overnight.
13. Next day, transfer reaction to 85 °C for 3 min. Afterward, place immediately on ice for 1 min.
14. 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 min.
15. Purify RNA, using sufficient ethanol to precipitate the ~150-nucleotide RNA fragments. For example, use an RNeasy Mini column (maximum capacity 100 μg RNA): 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 s at >8,000 × *g*. Discard flow-through and repeat with remaining sample. Add 500 μL buffer RPE to column and spin for 15 s. Discard flow-through. Repeat wash step. Transfer column to fresh collection tube and spin for 2 min to remove residual buffer RPE. Transfer column to 1.5 mL tube; add 30 μL H_2O and spin for 1 min to elute.
16. Measure RNA yield with a NanoDrop and dilute to a convenient concentration (e.g., 1 μg/μL). Ideal yield is >50 μg for a 40 μL reaction.
17. Run RNA on gel to confirm the correct size of the in vitro transcription product.

18. To generate ssDNA, set up a 200 μL reverse transcription reaction:
    - 120 μL RNA template (10 μg) + H_2O.
    - 20 μL 100 μM 5′-biotinylated Left Tag Primer.
    - 20 μL 10× AffinityScript buffer.
    - 20 μL 100 mM DTT.
    - 8 μL 100 mM dNTP (25 mM each).
    - 10 μL AffinityScript reverse transcriptase enzyme.
    - 2.5 μL RNase inhibitor.
19. Incubate at 55 °C for 50 min, then 75 °C for 5 min.
20. To degrade RNA templates, add 0.1× (20 μL) 1 M NaOH. Incubate at 75 °C for another 10 min.
21. Add 0.1× (20 μL) 1 M acetic acid to neutralize.
22. 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 min. Discard flow-through and repeat with remaining sample. Add 400 μL RNA Prep Buffer; spin for 30 s 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 min. Transfer column to a 1.5 mL tube, add 30 μL of water, and spin at 10,000 × *g* for 1 min to elute.
23. Measure the yield with a NanoDrop. Ideal yield is ~3 μg ssDNA for a 200 μL reverse transcription reaction.
24. Freeze biotinylated ssDNA probe until use.

3.4 Cell Harvesting and Cross-Linking

This protocol describes the steps for adherent cells. To adapt for suspension cells, spin cells between steps and decant supernatant to exchange buffers.

1. Before starting, heat one aliquot PBS at 37 °C and chill one aliquot at 4 °C (*see* below for volumes).
2. Grow adherent cells on 15-cm tissue culture plates. Before cross-linking, carefully split and count one plate (*see* **Note 6**).
3. Resuspend 50 mg of disuccinimidylglutarate (DSG) in 306 μL room temperature DMSO to create a 0.5 M stock solution.
4. Dilute DSG to 2 mM in room temperature 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 min to cross-link (*see* **Note 7**).
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 rinse once with room temperature PBS.
9. Add 7 mL warmed 3 % formaldehyde solution to cells. Incubate at 37 °C for 10 min, gently rocking by hand every 3 min.
10. Quench formaldehyde cross-linking by adding glycine to a final concentration of 500 mM. Incubate at 37 °C for 5 min.
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 1,000 × *g* at 4 °C for 5 min 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 min.
18. Remove supernatant and flash freeze pellets in liquid nitrogen. Store until cell lysis at −80 °C.

3.5 Cell Lysis

All steps and buffers should be cooled to 4 °C.

1. Thaw cell pellets by completely resuspending 20 million cells in 1 mL cell lysis buffer (add TCEP and PMSF fresh) in a 1.5 mL microcentrifuge tube.
2. Pellet cells by spinning at 3,300 × *g* for 7 min. Remove supernatant.
3. Gently resuspend swelled cells in 1 mL ice-cold cell lysis buffer premixed with 0.1 % NP-40. Incubate on ice for 10 min.
4. Transfer to an ice-cold glass dounce of appropriate size (e.g., 2 mL). Homogenize cell lysate by douncing 20×.
5. Transfer cells back to a microcentrifuge tube and pellet nuclei by spinning at 3,300 × *g* for 7 min. Remove supernatant.
6. Resuspend nuclei in 1 mL of nuclear lysis buffer (add TCEP and PMSF fresh). Incubate on ice for 10 min.
7. Sonicate using a Branson Sonifier fitted with a microtip using 5 W of power for 2 min in pulses: 0.7 s on, 3.3 s off (*see* **Note 8**).
8. Split sample into two separate microcentrifuge tubes, each with 500 μL lysate.

9. To each, add 6 μL of 100× DNase cofactor solution and 50–100 μL TURBO DNase (*see* **Notes 9** and **10**). Mix by pipet.
10. Transfer to a 37 °C heat block and incubate for 10–15 min (*see* **Note 10**).
11. Return sample to ice and immediately halt DNase reaction by adding 24 μL of DNase stop solution. Mix immediately by pipetting.
12. Remove and save a 5 μL aliquot of lysate (preclear).
13. Mix ~600 μL of lysate with 1.5 mL of 1.4× concentrated hybridization buffer.
14. Clear lysate by spinning at maximum speed (16,000 × *g*) for 10 min.
15. Remove and save a 5 μL aliquot of lysate (post-clear).
16. Flash-freeze aliquots of lysate in liquid nitrogen and store at −80 °C.
17. 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 (*see* **Note 11**), and incubate at 65 °C for 60 min.
18. Clean and purify nucleic acids as desired (e.g., ethanol precipitation).
19. Split in half; treat half with DNase and half with RNase, and incubate for 37 °C for 10 min.
20. Clean and purify nucleic acids. Elute in 10 μL H_2O.
21. Quantify DNA yields with a NanoDrop spectrophotometer (*see* **Note 12**).
22. Assess fragment sizes with the Agilent Bioanalyzer (High-Sensitivity DNA and RNA Pico kits). Ideal fragment sizes (preclear) are shown in Fig. 1.

3.6 Lysate Preparation

1. Thaw lysate corresponding to five million cells for each sample (*see* **Note 13**).
2. Aliquot out 100 μL MyONE streptavidin C1 magnetic beads for each purification from five 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 min 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 five million cells).
5. Incubate at 37 °C for 20–30 min, shaking.
6. Magnetically separate and transfer supernatant (streptavidin-cleared lysate) to a clean tube. Repeat this step to completely remove beads.

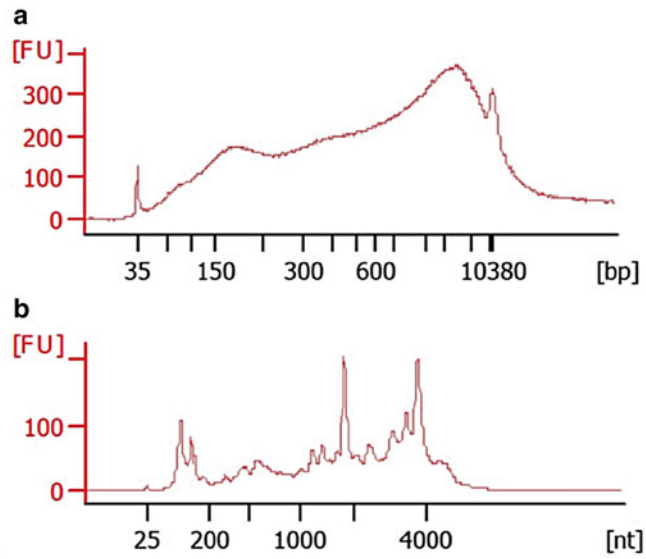

Fig. 1 DNA and RNA fragment sizes after lysis. (**a**) Example distribution of DNA fragment sizes (preclear). Sizes are measured with the High-Sensitivity DNA Bioanalyzer assay. (**b**) Example distribution of RNA fragment sizes (preclear). Sizes are measured with the RNA 6000 Pico Bioanalyzer assay. FU = fluorescence units measured by Bioanalyzer

7. Save 5 μL of precleared lysate (0.5 % total input) on ice as DNA input.
8. Rewarm lysate to 37 °C, then proceed immediately to hybridization.

3.7 Hybridization, Capture, Wash, and Elute

Prepare wash and elution buffers beforehand. Equilibrate solutions to the indicated temperatures before adding to samples.

1. Aliquot out 50 pmol of biotinylated ssDNA probe for each purification from five million cells.
2. Denature probe in H_2O at 85 °C for 3 min and then transfer immediately to ice.
3. Add probe to lysate, mix, and immediately transfer to a 37 °C ThermoMixer.
4. Incubate at 37 °C for 2–3 h, shaking at 1,200 rpm.
5. Just before use, aliquot out 500 μL of streptavidin C1 magnetic beads for each sample. Wash twice in 0.5× bead volume hybridization buffer, then resuspend in 0.25× bead volume hybridization buffer. Add beads to sample and incubate at 37 °C for 15–30 min, shaking.
6. Magnetically separate and then remove supernatant. Optional: save supernatant for quality control steps (*see* **Note 14**).
7. Resuspend beads in 1× original bead volume (500 μL) wash buffer, then incubate at 45 °C for 3–10 min while washing other samples. Wash a total of six times for each sample. 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.

8. Wash with 1× bead volume RNase H elution buffer (add TCEP and detergents fresh).
9. Wash with 100 µL RNase H elution buffer (add TCEP and detergents fresh). Transfer samples to new tube before removing the final wash.
10. To elute, add 55 µL RNase H elution buffer and 7.5 µL RNase H to each sample.
11. Incubate at 37 °C for 30 min, shaking.
12. Remove and save eluate.
13. Add 62.5 µL hybridization buffer and incubate at 37 °C for 5 min, shaking.
14. Remove eluate and combine with previous eluate.
15. Magnetically separate the combined eluates once more and transfer to a new tube to remove any residual beads and attached ssDNA probe.
16. 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 (*see* **Note 15**); for example, use 50 µL for RNA analysis and 450 µL for DNA analysis.

3.8 RNA Analysis

1. Mix well, and incubate tubes for 1 h at 65 °C to digest protein and reverse formaldehyde cross-links.
2. Clean and purify nucleic acids (e.g., using Zymo RNA Concentrator-5 columns). Elute in 15 µL H_2O.
3. Add 2 µL 10× TURBO DNase buffer, 2 µL TURBO DNase to digest genomic DNA, and 1 µL exonuclease I to digest residual ssDNA probe. Incubate at 37 °C for 15 min.
4. Clean and purify nucleic acids (e.g., using Zymo RNA Concentrator-5 columns). Elute in 12.2 µL H_2O.
5. Set up reverse transcription reaction:
   - 12.2 µL RNA.
   - 2 µL 10× RT random primers.
   - 2 µL 10× AffinityScript buffer.
   - 2 µL 100 mM DTT.
   - 0.8 µL 100 mM dNTPs (25 mM each).
   - 1 µL AffinityScript RT enzyme.
6. Mix. Incubate at 25 °C for 10 min, 55 °C for 50 min, and 70 °C for 15 min.

7. Place samples on ice. Dilute cDNA by adding 180 μL H_2O.
8. Analyze cDNAs using quantitative real-time PCR to determine enrichment and yield of the target RNA (*see* **Note 16**). 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).
9. 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 in Subheading 3.6, **step 7**.

3.9 DNA Analysis

1. Mix well, and incubate tubes overnight at 60 °C to digest protein and completely reverse formaldehyde cross-links.
2. Clean and purify DNA.
3. If desired, analyze genomic DNA using quantitative real-time PCR to determine enrichment and yield of target DNA (*see* **Note 17**).
4. Generate DNA sequencing libraries for high-throughput DNA sequencing using the NEBNext Ultra DNA Library Prep Kit for Illumina using the manufacturer's protocol. Sequence the DNA libraries to generate ~20 million reads for the RAP samples and 30–80 million reads for the input samples (*see* **Note 18**).

4 Notes

1. As a positive control, we suggest purifying the highly abundant U1 snRNA. In mouse cells (*Mus musculus*), use the following three 5′-biotinylated probes: (1) CAGGGGAGAGCGCGAA CGCAGTCCCCCACTACCACAAATTATGCAGTCGA, (2) GTTTCCCGCATTTGGGGAAATCGCAGGGGTCAGC ACACCCCAAAGTGCAA, and (3) TGGGTGAGCCTCGC CCTGGGAAAACCACCTTCATGATCATGGTATCTCCC.
2. Nonspecific hybridization of probes to off-target RNAs or DNA loci is a major concern for hybridization-based purification strategies. RAP uses highly denaturing and stringent hybridization conditions to ensure capture specificity. However, nonspecific interactions are 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.
3. Single-stranded DNA probes provide better specificity than the RNA probes used in previous iterations of this protocol [12].
4. An alternative strategy for obtaining RAP probes is to order 5′ biotinylated oligos from a commercial supplier. Compared

to the protocol presented above, 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.

5. In some cases, specific amplification of subsets of the oligo pool can be challenging, depending on the quality of the oligo synthesis and the complexity of the oligo pool. We typically order oligo pools with 12,000 unique sequences from Custom Array, Inc. and achieve robust amplification of full-length product.
6. 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 cross-link 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.
7. For some cell types, reducing the cross-linking amount (e.g., to 2 % formaldehyde) or reducing the length of the DSG treatment may allow for better lysis and solubilization.
8. 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.
9. TURBO DNase works better than standard DNase I here because it works more robustly in high-salt solutions.
10. 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 to 4 min, and DNase treatment might vary from 10 to 20 min, depending on cell number, ploidy, and cross-linking strength. To optimize DNase timing and conditions, remove 5 μL lysate aliquots every 2–4 min, quench with EDTA and EGTA on ice, and assay RNA and DNA sizes for each time point as described in the protocol. In optimizing the lysis conditions, the goals are (1) to solubilize as much chromatin as possible (assayed by comparing DNA concentrations from lysate before and after the clearing spin in Subheading 3.5, **step 14**), (2) to digest the DNA to appropriate fragment sizes for DNA sequencing (with a significant fraction of fragments in the range of 100–600 base pairs as assayed by gel electrophoresis or Bioanalyzer), and (3) to avoid severe degradation of cellular RNA (assayed by gel electrophoresis or Bioanalyzer). 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 cross-linking may be necessary.

11. The hybridization buffer contains 3 M guanidine thiocyanate, which precipitates sodium dodecyl sulfate (SDS) detergent; do not use SDS in buffers in place of *N*-lauroylsarcosine. High concentrations of guanidine thiocyanate at high temperature will denature dsDNA fragments: in order to avoid denaturing dsDNA, dilute final guanidine thiocyanate concentration eightfold (to less than 375 mM final concentration) during any high-temperature proteinase K digestion.
12. Accounting for the dilution of the lysate between the pre-clear and post-clear aliquots, we typically find that the post-clear aliquots contain >50 % of the DNA of the pre-clear aliquot, indicating successful solubilization of chromatin.
13. We have found that five million cells per sample is more than enough to map RNA-chromatin interactions for abundant noncoding RNAs like Xist or U1; for lower-abundance RNAs, more cells may be necessary. In this case, scale up probe amounts and reaction volumes accordingly.
14. 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 (1) the integrity of the RNA at the end of the hybridization, (2) the amount of target RNA/DNA remaining in the supernatant after capture, and (3) the amount of probe remaining in the supernatant after capture.
15. RNA analysis should be used initially to validate capture of the target RNA. After establishing the reproducibility of the assay, RNA analysis can be skipped and the entire sample can be used for DNA analysis.
16. RNA enrichments are typically in the range of 100- to 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 cross-linked macromolecular complexes are too large due to over-cross-linking or insufficient sonication; try decreasing cross-linking 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.
17. 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.

18. 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 [12].

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