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Publication Date
2017

DOI
10.1007/978-1-4939-6807-7_15

Peer reviewed

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Author manuscript
Methods Mol Biol. Author manuscript; available in PMC 2017 September 06.

Published in final edited form as:
Methods Mol Biol. 2017 ; 1562: 231–243. doi:10.1007/978-1-4939-6807-7_15.

High-throughput small RNA sequencing enhanced by AlkB-facilitated RNA de-Methylation (ARM-seq)

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Summary

N^1-methyladenosine (m[1]A), N^3-methylcytidine (m[3]C) and N^1-methylguanosine (m[1]G) are common in transfer RNA (tRNA) and tRNA-derived fragments. These modifications alter Watson-Crick base-pairing, and cause pauses or stops during reverse transcription required for most high-throughput RNA sequencing protocols, resulting in inefficient detection of methyl-modified RNAs. Here we describe a procedure to demethylate RNAs containing m[1]A, m[3]C or m[1]G using the *Escherichia coli* dealkylating enzyme AlkB, along with instructions for subsequent processing with widely used protocols for small RNA sequencing.

Keywords

RNA sequencing; transfer RNA (tRNA); AlkB; RNA demethylation; N^1-methyladenosine (m[1]A); N^3-methylcytidine (m[3]C); N^1-methylguanosine (m[1]G)

Introduction

High-throughput sequencing has become commonplace for characterizing small RNA populations, which can include microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), and transfer RNAs (tRNAs), as well as partial fragments of tRNAs and ribosomal RNAs (rRNAs). tRNAs in particular are highly modified, often containing multiple nucleoside modifications that interfere with Watson-Crick base-pairing and are known to block reverse transcription during cDNA synthesis [1,2]. Because most small RNA sequencing protocols are designed specifically to clone only full-length cDNAs rather than partial cDNAs derived from segments of larger RNAs, RNAs containing modifications that pause or block reverse transcription are typically underrepresented or absent in small RNA sequencing results.

One approach to facilitate sequencing of modified RNAs is to enzymatically remove problematic modifications prior to sequencing library preparation. The *Escherichia coli*

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AlkB protein is a dealkylating enzyme that has been shown to remove methyl groups from m^1A, m^3C and m^1G residues in RNA [3–5]. Purified AlkB enzyme was used previously to identify AlkB-sensitive m^3C residues in tRNAs in primer extension assays [6]. We subsequently adapted the approach for demethylation pre-treatment to facilitate high-throughput sequencing of m^1A, m^3C or m^1G modified small RNAs, revealing an abundance of methyl-modified tRNA-derived small RNAs [7]. Here we describe procedures for overexpression and purification of the AlkB enzyme, small RNA isolation, and sequencing library preparation. The small RNA library preparation kit used here is NEBNext, produced by New England Biolabs. Similar results have been obtained using TruSeq small RNA kits from Illumina. Specific RNAs that are methylated can be subsequently identified by comparing sequencing results from control (buffer-treated) versus demethylated RNA using statistical methods for differential abundance analysis such as DESeq2 [8]. Our lab has implemented specialized tRNA comparative read analysis and visualization in a freely available software package (http://trna.ucsc.edu/ARM-seq/).

2. Materials

Use nuclease-free reagents for all buffers and decontaminate all equipment using RNA-Zap and 70% ethanol prior to starting any step. Use nuclease-free low-binding microcentrifuge tubes to maximize yield at each step. Cloning of the *E. coli* AlkB gene into the AVA421 plasmid was described by Quartley et al [9]. *E. coli* BL21(DE3)pLysS competent cells (Promega) were transformed with AlkB-AVA421 plasmid according to manufacturer's protocol (any equivalent *E. coli* competent cells commonly used for protein expression may be used). Cells were flash frozen on dry ice and stored at −80°C.

2.1 Purification of recombinant AlkB

1. Frozen cell pellet from 12 L culture of AlkB-AVA421-transformed cells grown at 37°C to OD^{600} 0.5 and induced with IPTG for 2 h.
2. Sonication buffer: 20 mM Hepes pH 7.5, 5% glycerol, 1 M NaCl, 2 mM β-mercaptoethanol (BME), 2 μg/mL Leupeptin, 1 μg/mL Pepstatin, 1 mM Pefabloc
3. TALON Metal Affinity Resin, 2 mL per liter of bacterial culture.
4. 0.5 M NaCl Wash Buffer: 20 mM Tris-HCl pH 8.0, 5% glycerol, 2 mM BME, 0.5 M NaCl.
5. 0.8 M NaCl Wash Buffer: 20 mM Tris-HCl pH 8.0, 5% glycerol, 2 mM BME, 800 mM NaCl.
6. No Salt Buffer: 20 mM Tris-HCl pH 8.0, 5% glycerol, 2 mM BME
7. E1 buffer: 5 mM imidazole dissolved in 0.5 M NaCl Wash Buffer
8. E2 buffer: 10 mM imidazole dissolved in 0.5 M NaCl Wash Buffer
9. E3 buffer: 250 mM imidazole
10. Bradford assay components (Bio-Rad Catalog)
11. HRV 3C protease (Clontech), or equivalent 3C protease

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12. Dialysis Buffer: 20 mM Tris-HCl pH 8, 5% glycerol, 200 mM NaCl, 2 mM BME.
13. HiLoad 16/60 Superdex 200 gel filtration column.
14. HiLoad Buffer: 20 mM Tris-HCl pH 8, 5 % glycerol, 500 mM NaCl, 2 mM DTT, 0.025% NaN_3.
15. Dialysis cassette 10,000 MWCO (Thermo Scientific Pierce Slide-A-Lyzer cassette)
16. Storage buffer: 20 mM Tris-HCl pH 8.0, 50% glycerol, 200 mM NaCl, 2 mM DTT.
17. 8–16% SDS polyacrylamide gel (SDS-PAGE), Tris-HCl buffered, 18 well.
18. Glass chromatography column, 1.5 x 10 cm, max volume 18 mL (e.g., BioRad Econo-Columns, 1.5 x 10cm).

2.2 RNA Isolation

1. Tri-Reagent, used to harvest and freeze down mammalian cells for storage in −80°C until RNA isolation.
2. Direct-Zol TM RNA MiniPrep Kit (Zymo Research) for RNA extraction, with Pre-wash buffer.
3. mirVana miRNA Isolation kit without phenol (Ambion) for selection of RNA <200nt.
4. BioAnalyzer Small RNA kit (Agilent Technologies) for assessment of quality and sizes of RNA input into library synthesis.

2.3 DNase Treatment

1. DNase I, 2000 U/mL, supplied with 10x reaction buffer
2. 0.5 M EDTA, pH8.0
3. RNA Clean & Concentrator (Zymo Research) for RNA clean up

2.4 AlkB Demethylation

1. 1. 2x common mix, 50μL made directly before use by adding 10 μL of each of the following stock solutions on ice:

   500mM Hepes-KOH, pH 8.0

   750mM Ferrous Ammonium Sulphate pH 5.0

   10mM Alpha-ketogluterate in 50mM Hepes-KOH pH 8.0

   20mM Na-Ascorbate (made fresh, and shielded from light)

   500μg/mL BSA

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2.5 cDNA library preparation, size selection, and analysis

1. NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs) for preparation and indexing of cDNA libraries.
2. E-Gel SizeSelect™ Agarose Gels, 2% (Invitrogen) for size selection of libraries.
3. E-Gel 50 bp DNA Ladder (Invitrogen) for guiding the size selection of libraries.
4. 10 mM Tris,1 mM EDTA, pH 8.0 (TE)
5. DNA Clean and Concentrator-5 kit (Zymo Research) for clean up steps.
6. Agilent High Sensitivity DNA Kit (Agilent Technologies)

3. Methods

3.1 Purification of recombinant AlkB

Carry out all parts of this protocol at 4°C in a cold room. All centrifugation steps were performed with a swing bucket rotor, except as noted in step 1. Equivalent RCF x g r_{av} values given.

1. Thaw cell pellet from cells expressing AlkB, resuspend in 240 mL sonication buffer (12 L cell culture x 20 mL buffer per liter of culture), sonicate 8 times for 10 seconds with a probe sonicator (each time followed by one minute on ice), and remove debris by centrifugation at 10,100 RCF r_{av} (e.g., we use 10,000 rpm in a Beckman Coulter JLA-16.250 fixed angle rotor). Carefully remove clarified supernatant crude extract ("CE") to a clean tube. It can be used immediately or flash frozen on dry ice for storage at −80°C until needed. Discard cell debris pellet.
2. Prepare 24 mL TALON resin (2 mL resin per L of cell culture) by washing three times with 0.5 M NaCl Wash Buffer. Use 25 mL Wash Buffer per 2 mL resin, centrifuge at 700 RCF for 4 minutes (e.g., 2000 rpm in a Beckman Coulter JS-5.3 swing bucket rotor). Remove and discard supernatant after each centrifugation.
3. Add 240 mL of No Salt buffer to 240 mL of CE. Save 10 μL of diluted CE for SDS-PAGE.
4. Divide the diluted CE into 12 x 50 mL conical tubes (40 mL per tube), each containing 2 mL of prewashed resin.
5. Mix slowly for 1 hour (e.g., on an orbital shaker) to allow his-tagged protein to bind resin.
6. Spin at 1400 RCF for 5 minutes (e.g., 4000 rpm in JS-5.3 swing bucket rotor.
7. Carefully remove as much supernatant as possible without disturbing resin pellet. Keep resin (with bound protein), but save 10 μL of flow-through ("FT") supernatant for SDS-PAGE. (Optionally, keep all of supernatant, temporarily, until protein recovery is confirmed at end of isolation.)

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8. Consolidate 12 tubes of resin into two tubes by adding 25 mL 0.5 M NaCl Wash Buffer in each of two tubes, and combing resins across six total tubes (leaving two final tubes with approximately 12 mL of combined resin and 25 mL of wash buffer per tube).
9. Mix gently for 10 minutes.
10. Spin at 1400 RCF for 5 minutes.
11. Remove and discard supernatant.
12. Repeat wash procedure three times: add 25 mL 0.5 M NaCl Wash Buffer to each of two tubes, mix for 10 minutes, spin at 1400 RCF for 5 minutes, and discard supernatant.
13. Add 25 mL of E1 buffer to the resin.
14. Mix gently for 10 minutes.
15. Spin at 1400 RCF for 5 minutes.
16. Remove and discard supernatant as Eluent 1 (“E1”). Optionally, keep 10μL for SDS-PAGE analysis.
17. Add 15 mL of E2 buffer to each of 2 tubes, and mix gently.
18. Combine all of sample (E2 buffer and all resin from both tubes) by pouring slowly into one glass chromatography column.
19. Collect drip flowthrough into a 50mL beaker (don’t let the resin dry out) as Eluant 2 (“E2”).
20. Add 10 mL more of E2 buffer and let drip into same 50 mL beaker. Protein should still be bound to resin in column. (Optionally, keep all of E2 in beaker, temporarily, until protein recovery is confirmed at end of isolation.)
21. To elute the bound protein, add 30 mL E3 buffer to the column (add buffer slowly, only 10 mL at a time).
22. Collect the first eight 3 mL fractions (“E3 fractions”) in individual tubes.
23. Do a Bradford assay on all eight collected E3 fractions, and combine only fractions with most protein (generally two or three fractions, so final volume will be 6–9 mL of eluted protein).
24. Quick freeze samples collected for SDS-PAGE: 10 μL diluted CE, 10 μL FT, and 4 μL E3 (and 10 μL optional samples of E1 and E2) and store at −70°.
25. Calculate the amount of HRV 3C protease needed and add to pooled E3 protein fractions from step 23. (1:75 by mass, eg., 1 mg protease/75 mg protein).
26. Dialyze in dialysis cassette into 2 L of Dialysis Buffer overnight (*see* Note 1).

[1]200 mM NaCl dialysis buffer enhances efficiency of 3C protease cleavage.

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27. Next Day: harvest dialyzed protein supernatant ("DP") from dialysis cassette. If supernatant is cloudy from a small amount of protein that has precipitated during dialysis, remove precipitate by dividing DP sample into 2 mL microfuge tubes and spin for 1 minute at 1400 RCF, and transfer supernatant (with protein) to new tube, discarding pellet.
28. Take a sample of DP (4 µL) for SDS-PAGE analysis and determine protein concentration by Bradford assay.
29. Prepare fresh TALON resin by combining 1.5 mL resin slurry and 10 mL of 0.5 M NaCl Wash buffer in a 15 mL conical tube. Gently mix and spin at 1400 RCF for 5 minutes to pellet resin. Discard the supernatant and repeat the wash twice.
30. Dilute DP (should have 5–10 mL from step 27) with an equal volume of 0.8 M NaCl Wash Buffer (to bring NaCl back up to 0.5 M for TALON binding), and move to 50 mL conical tube.
31. Add 0.75 mL washed TALON resin to diluted DP (this step binds HRV 3C protease, and any other non-AlkB contaminant proteins to TALON resin).
32. Mix gently for 1 hour.
33. Spin down the resin at 1400 RCF for 5 minutes.
34. Remove and save the protein supernatant ("PS"), discard resin pellet.
35. Do Bradford assay on PS, and keep 8 µL for SDS-PAGE.
36. Run SDS-PAGE on all samples previously saved for gel analysis (CE, FT, E3, DP, PS) for 1 hour at 200 V and then stain with Coomassie blue.
37. Concentrate down the entire PS from step 33 to 5 mL or less for sizing column. If sample is cloudy, spin sample for 5 minutes at 1400 RCF, and discard pellet, keeping supernatant, as in step 27. Perform Bradford assay to determine protein concentration. Save 20 µL of the concentrated sample ("CS") for SDS-PAGE analysis.
38. Load all of CS on the HiLoad 16/60 Superdex 200, 120 mL column in HiLoad Buffer (pre-equilibrated with 5 column volumes of HiLoad buffer), and collect fractions of 3 mL overnight.
39. Run SDS-PAGE analysis, as in step 35, of 20 µL aliquots of all sizing column fractions, combine peak fractions (about 3–5 fractions) and perform Bradford assay of the pooled sample.
40. Concentrate down to appropriate volume (0.5–3 mL) for dialysis cassette. Save 10 µL sample for SDS-PAGE.
41. Dialyze sample overnight into 2 L Storage Buffer, overnight.
42. The next day, collect protein from the dialysis cassette. If the solution is cloudy, spin down, discard pellet as in step 27. Keep supernatant and transfer to a new tube. (*see* Note 2)

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43. Determine protein concentration by Bradford assay and take a 2–20 μL sample (20 μg) for SDS-PAGE.
44. Run SDS-PAGE analysis, as in step 35, with different concentrations and size standards on gel to assess purity (see example SDS-PAGE gel in Figure 1; should see a single clear band of ~26 kDa, with only minor contaminants visible in the 10 μg sample).
45. Aliquot ¾ of the final dialyzed protein into small aliquots, freeze on dry ice and store at −80°C.
46. Store remaining ¼ of protein and 5 aliquots of 1 mL of storage buffer (to use as enzyme dilution buffer later, or in control "no enzyme" ARM-seq experiments) at −20°C for use within 60 days (AlkB activity is retained for at least 60 days at −20°C).

3.2 Total RNA isolation from mammalian cells frozen in Tri-Reagent

1. Remove two cryovials of 10^7 cells each frozen down from an 80–90% confluent culture in 1 mL of Tri-Reagent and place on ice. Work with Tri-reagent should be done in the fume hood and waste should be disposed of appropriately, to avoid phenol and guanidinium thiocyanate exposure and contamination.
2. While thawing on ice, pipet up and down to quickly expose cell contents to Tri-Reagent to limit RNA degradation.
3. Prepare two columns from the DirectZol kit for each cryovial (four total).
4. Vortex the thawed Tri-Reagent cell extracts vigorously for 10 seconds.
5. Incubate the vials for 5 minutes at room temperature.
6. Transfer to nuclease free microcentrifuge tubes and spin out debris for 1 minute at 12,000 RCF.
7. Transfer supernatant to clean tubes and add 1 mL 100% ethanol.
8. Vortex for 10 seconds before distributing 700 μL of lysate from each tube over the four prepared columns.
9. Spin loaded columns for 1 minute at 12,000 RCF and discard flow through.
10. Repeat previous step until all material is loaded on columns (*see* Note 3).
11. Wash columns twice with 400 μL of DirectZol kit Prewash buffer and once with 700 μL of DirectZol kit Wash buffer as directed by DirectZol miniprep kit instructions.
12. Remove residual DirectZol kit Wash buffer with a final 2 minutes spin at 12,000 RCF and discard flow through.

[2]In-column DNaseI treatment using DirectZol kit-provided components and instructions can be performed at this step.
[3]AlkB protein has "peachy" color to it.

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13. Elute RNA from each column into a clean tube with 25 μL of nuclease free water.

14. Remove 1 μL for 1:10 dilution in nuclease-free water for concentration determination using a NanoDrop spectrophotometer before storing purified total RNA at −80°C.

15. Expected yield from each cryovial of 10^7 cells is 30 μg (combined total of 60 μg total RNA available at this step).

3.3 Size Selection for <200nt RNA using MirVana Kit (see Note 4)

1. Raise volume of total RNA solution from step 13 of section 3.2. to 150 μL by adding nuclease free water.

2. Prepare two columns from MirVana miRNA Isolation Kit following the manufacturer's instructions.

3. Preheat Elution solution to 95°C.

4. In a fume hood, add 5x volume of Lysis/Binding Buffer containing the denaturant guanidinium thiocyanate, a potential hazard, (750 μL to 150 μL of RNA suspension) and briefly vortex.

5. Add 1/10th volume of miRNA Homogenate Additive (90 μL to 900 μL from the above step) and briefly vortex.

6. Place on ice for 10 minutes.

7. Add 1/3 volume of 100% ethanol (330 μL to 990 μL from above step) and vortex briefly.

8. Filter the entire sample from the step above through one column by adding 700 μL at a time and spinning 5,000 RCF for 1 min. Repeat until the entire volume has been applied to the column. The flow-through contains the <200nt fraction (*see* Note 5).

9. Combine flow-through (~1220 μL total) and split into two nuclease-free microfuge tubes (~610 μL each).

10. Raise the ethanol concentration of the flow through to promote column binding of the smaller RNA by adding 2/3 volume of 100% ethanol (~406.6 μL) to each tube and vortex briefly.

11. Load the contents of both tubes on a single fresh column by repeatedly adding 700 μL at a time and spinning for 1 minute as above. The flow through can be discarded each time because the <200 nt RNA is bound to the column by this step.

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[4]To facilitate calculation of reagent volumes needed for processing of multiple samples, RNA size selection and all subsequent processing steps as described are scaled for preparation of a single sample. Scale volumes appropriately to include all sample replicates and untreated controls. Be sure to follow manufacturer's instructions and dispose of kit components appropriately.
[5]This step binds large RNAs to the column, allowing the <200nt RNA to flow through.

12. Wash the column with 700 μL of Wash 1 followed by two washes of 500 μL Wash 2/3 each and spinning as above according to kit instructions.
13. To dry the column, spin empty at 10,000 RCF for 1 min.
14. Transfer column to clean, low-binding, nuclease-free tube and add 50 μL warmed Elution Solution provided in the kit
15. Incubate for 2 minutes at room temperature before spinning as above to elute the RNA.
16. Repeat steps 14–15. Final volume of <200 nt RNA is 100 μL.
17. Expected yield at this step is 20–30% of the amount of input RNA (expect 12–15 μg of <200 nt RNA).

3.4 DNAse I treatment and Clean-up of <200 nt RNA (see Note 6)

1. Combine <200 nt RNA from above and place 90 μL into each of two thin-walled 0.2 mL PCR tubes.
2. Add 10 μL of 10X DNase I reaction buffer.
3. Add 1 μL of DNase I.
4. Incubate in thermocycler at 37°C for 10 minutes.
5. Add 1 μL of 0.5 M EDTA and incubate at 75°C for 10 minutes.
6. Combine DNase I reactions and clean and concentrate RNA by following directions in the RNA Clean and Concentrator-5 kit.
7. Elute RNA from one column using 17 μL of nuclease-free water.
8. Expected yield at this step is 85% of input RNA (expect 10–12 μg of <200nt DNAse-free RNA at approximately 0.6–0.7μg/μL available at this step).

3.5 Demethylation of RNA by AlkB Treatment

Equal amounts (w/w) of ALKB enzyme and RNA are used in the demethylation reaction. For use in the protocol below adjust purified ALKB to a concentration of 15.4 ug/μL. Control samples used for differential abundance analysis should be treated with AlkB Storage buffer only, from step 45 of Section 3.1 (no enzyme addition, *see* Note 7).

1. Immediately before use, prepare 50 μL of a 2x common mix on ice:
2. In a thin-walled 0.2 mL PCR tube, add the following:

   15 μL of <200nt RNA

   0.65 μL of 15.4μg/μL AlkB (or AlkB buffer only for control samples)

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[6]DNase treatment and clean-up can now be performed at the RNA extraction step with an in-column protocol sold with newer versions of the Direct-Zol RNA extraction kit (see Note 2).

[7]Control reactions are treated with AlkB buffer in order to verify that observed changes in sequencing are specific to AlkB enzyme activity rather than potential changes in modification state or cloning efficiency due to buffer incubation conditions.

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4.35 µL of water

20 µL of 2X common mix

3. In a thermocycler, incubate the reaction at 37°C for 100 minutes.
4. Stop and clean up reaction using Zymo Research RNAcc-5 cleanup, eluting with 10 µL of ddH_2O.
5. Measure RNA concentration using a NanoDrop spectrophotometer. Check the quality of the RNA using Agilent Small RNA kit for the BioAnalyzer and 1:20 dilutions of the RNA samples (Figure 2). The typical yield at this step is 4–6 µg of <200 nt demethylated DNA-free RNA in 8µL of water.

3.6 Small RNA Library Preparation and Size Selection of Libraries

(for guidance on sequencing platforms and read processing see Notes 8 & 9).

1. Typically 1 µg RNA from the above is used as input into the NEBNext (or comparable) small RNA library construction kit, and instructions are followed with the exception of 60°C temperature used for the reverse transcription.
2. After 15 cycles of PCR, clean samples and concentrate using the DNA Clean and Concentrator-5 kit, eluting with 27 µL of Elution buffer provided with the kit. At this point, samples can be stored at −20°C until size selection of the libraries.
3. To size select the adapted libraries from adaptor-adaptor ligation products, load samples on pre-rinsed 2% Agarose SizeSelect E-gels with a 50 bp E-gel ladder and run for 10 minutes. Rinse the lower wells and image the gel. Then continue electrophoresis and withdraw 20 µL every 10–12 sec and replace with fresh TE starting once the 100 bp band of the ladder passes from the bottom well and finishing as the 250 bp band clears the well. Typically, 350 µL are collected per sample (Figure 3).
4. Purify the size-selected libraries using the DNA clean and concentrator-5 kit and elute with 18–20 µL TE.
5. BioAnalyzer traces using the Agilent DNA High Sensitivity kit are done on 1:10 dilutions of the libraries to assess the amount of adaptor-adaptor product at 120 bp and for concentration determinations (Figure 4).
6. Samples are ready for indexing, pooling, and sequencing, following library kit instructions.

[8]For good sensitivity aim for at least 10–20 million reads per sample, which gave good results for resolving methyl-modified tRNA fragments. 12–18 indexed samples can be pooled per lane when using the Illumina MiSeq platform. HiSeq allows for additional pooling.

[9]Sequencing reads derived from tRNAs should be processed with specialized software to appropriately map transcripts and fragments that contain posttranscriptional modifications common in mature tRNAs (e.g. 3'-CCA ends). Software designed for this purpose is freely available (see http://trna.ucsc.edu/ARM-seq/). Examples of ARM-seq sequencing results mapped to individual tRNAs can be viewed in the Genomic tRNA Database (http://gtrnadb.ucsc.edu) by navigating to any individual tRNA gene entry for *Homo sapiens* (GRCh37/hg19) or *Saccharomyces cerevisiae* S288c, and scrolling to the bottom of the page to view "Expression Profiles".

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Acknowledgments

This work was supported by the US National Institutes of Health (NIH) NHGRI grant 5R01HG006753 to T.M.L. and by NIH grant GM052347 to E.M.P. Thanks to Patricia Chan for assistance in preparation of figures.

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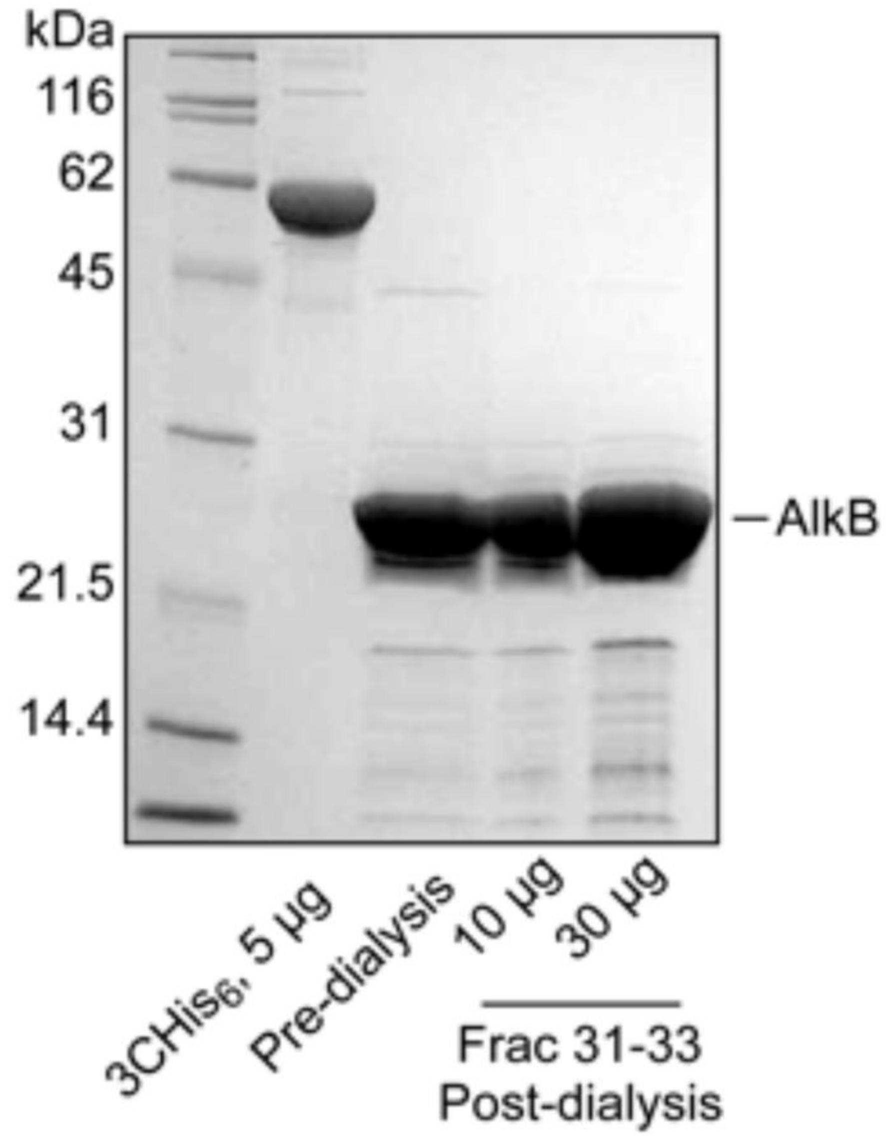

Figure 1.
Typical SDS-PAGE analysis of purified AlkB protein.

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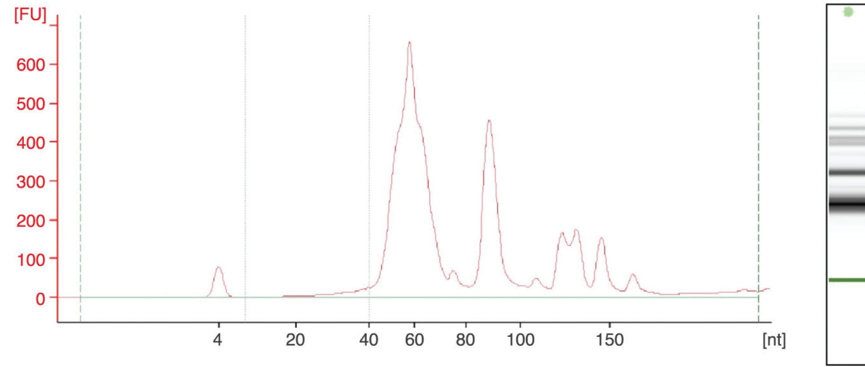

Figure 2.
Typical BioAnalyzer Trace of human small RNA preparation after DNase and AlkB treatment, 1:20 dilution.

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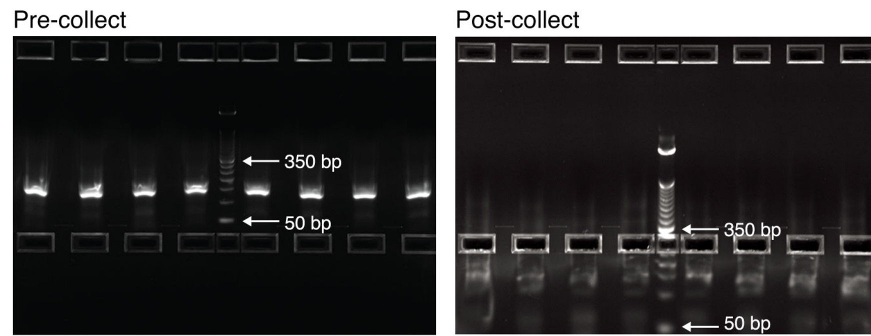

Figure 3.

Typical size selection of cDNA libraries adapted for Illumina sequencing using 2% Agarose Size Select E-gel. Photographs are taken 10 minutes after starting the run (a), and after the collection of the desired fraction (b). A 50 bp E-gel ladder was used as a guide for fraction collection.

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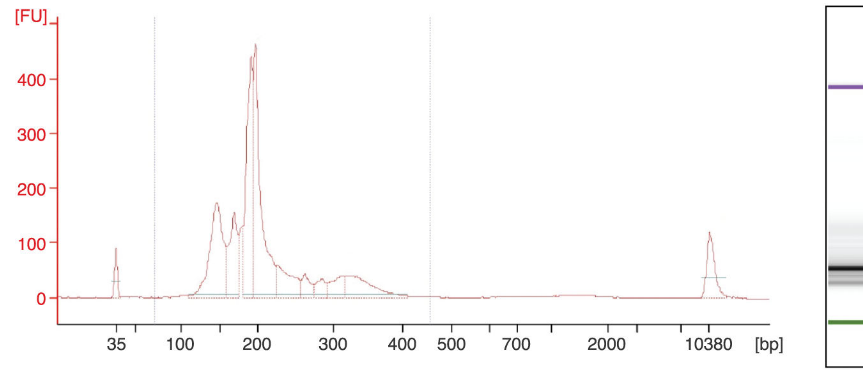

Figure 4.
Typical BioAnalyzer trace of 1:10 dilution of cDNA library used as input for Illumina sequencing in ARM-seq.