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Identifying Argonaute binding sites in *Caenorhabditis elegans* using iCLIP

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Abstract

The identification of endogenous targets remains an important challenge in understanding microRNA (miRNA) function. Past approaches using *in silico* methods and reporter constructs lack biological context that may enhance or inhibit target recognition. To address these limitations, several labs have utilized crosslinking and immunoprecipitation (CLIP) of Argonaute (Ago) proteins to identify miRNA targets. Recently, the Ule Lab introduced individual-nucleotide resolution CLIP (iCLIP) to increase the sensitivity of identifying protein-RNA interaction sites. Here we adapt the iCLIP protocol for use in *Caenorhabditis elegans* to identify endogenous sites targeted by the worm Argonaute (ALG-1) primarily responsible for miRNA function.

Keywords

Argonaute; iCLIP; miRNA; *C. elegans*

1.1 Introduction

In many eukaryotic organisms, small RNA molecules, known as microRNAs (miRNAs), provide post-transcriptional control of gene expression through imperfect binding to target RNAs. This flexibility in targeting allows each miRNA to potentially regulate hundreds of different transcripts in a range of pathways [1]. As a consequence of their broad regulatory potential, altered miRNA expression is implicated in many diseases [2].

The majority of miRNA biogenesis begins with transcription of a primary-miRNA (pri-miRNA) transcript by RNA polymerase II [3]. This long, poly-adenylated transcript is capable of forming an imperfectly paired stem-loop structure and is processed by Drosha and DGCR8/Pasha into a ~65 nucleotide (nt) long hairpin precursor-miRNA (pre-miRNA). Maturation of the miRNA occurs after subsequent Dicer processing of the pre-miRNA into its final ~21 nt form. Mature miRNAs are loaded onto an Argonaute (Ago) protein as part of the miRNA Induced Silencing Complex (miRISC). Classically, miRNAs are thought to help direct the miRISC to the 3'UTR of messenger RNAs (mRNAs), resulting in a decrease in gene expression through mRNA destabilization and/or translational repression [1]. More recently, it has been shown that miRNAs can target regions throughout mRNAs [4–6] and non-coding RNAs [7].

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Over the past decade, one of the greatest challenges in the field has been identifying miRNA target sites. Predicting targets is complicated by the limited sequence information provided by miRNAs due to their small size and their ability to function even when only a partial duplex is formed [1]. In these cases, as few as 7 nts can provide functional targeting information to the miRISC [8]. *In silico* approaches are often based on common motifs, such as the “seed” sequence found in nucleotides 2–7 of the miRNA, and the propensity for miRNAs to bind to the 3'UTR of messenger RNAs. However, these programs often miss non-canonical target sites and have high rates of false positives, as the presence of a binding site does not ensure regulation. Furthermore, there is little consensus among miRNA target prediction programs. To address these problems, several labs have utilized high-throughput sequencing following ultraviolet (UV) crosslinking and immunoprecipitation (CLIP-seq or HITS-CLIP) and other similar methods to identify Argonaute/miRNA binding sites [4–6,9–11]. In HITS-CLIP, target RNAs are identified by first crosslinking with UV light, which generates a covalent bond between the protein of interest and the RNA. These protein-RNA complexes are then immunoprecipitated. By subsequently adding 5' and 3' adapters and removing the protein through Proteinase K digestion, the isolated target RNAs are reverse-transcribed, amplified, and identified through high-throughput sequencing. These studies have revealed the prevalence of non-3'UTR binding sites in target mRNAs [4–6], new binding motifs [12], and have the potential to improve *in silico* target prediction approaches.

Since its development, HITS-CLIP has become increasingly used for determining target sites for a range of RNA-binding proteins, particularly in the fields of splicing and small RNA research. At first, miRNA target site identification was limited to the size of the RNA fragment isolated after RNA trimming and immunoprecipitation. However, analysis of sequencing data revealed that mutations and deletions occur during generation of the cDNA library at the crosslinking site due to the presence of an amino acid or short peptide that remains attached after Proteinase K treatment. From these data it is possible to determine RNA-protein interaction locations with increased resolution [13,14]. An adaptation on HITS-CLIP, known as photoactivatable-ribonucleoside-enhanced (PAR)-CLIP, uses photoreactive ribonucleoside analogs and UV-A light to increase crosslink efficiency and the incidence of point mutations at the crosslinking site [6]. However, PAR-CLIP requires tissues and cells to be pre-treated with the photoreactive ribonucleoside analogs before crosslinking. This may result in toxic effects [15] that may alter endogenous conditions. Furthermore, primer extension studies and analysis of HITS-CLIP data have demonstrated that a significant portion of reverse transcription products terminate at the site of crosslinking [16,17].

To address these problems the Ule Lab developed a new method known as individual-nucleotide resolution CLIP (iCLIP) [18,19]. In brief, iCLIP is designed to isolate all cDNA products generated during reverse transcription, including cDNAs that truncate at the crosslinking site. Additionally, iCLIP uses barcoded primers during generation of the cDNA library to control for PCR artifacts that may occur with library amplification [20]. A recent analysis of HITS-CLIP and iCLIP datasets has revealed that 80% of cDNAs truncate at the crosslink site and are absent in HITS-CLIP data [17]. This analysis also showed that the use of RNase I, which can cleave between any ribonucleotide pairs, in iCLIP alleviates the bias in target site identification that results from the use of other enzymes during RNA trimming. These advantages allow iCLIP to recover additional reads and provide increased confidence in the identification of bona fide target sites.

As a complement to both HITS-CLIP and PAR-CLIP, which have been previously adapted for use in *Caenorhabditis elegans* [5,21], we have developed a procedure to bring the advantages of the iCLIP protocol [19] to *C. elegans* for the identification of Argonaute/miRNA regulated RNAs (Figure 1). For this application *C. elegans* offers several

advantages: (1) Argonaute-like-gene 1 (ALG-1) is largely responsible for miRNA function, (2) crosslinking in a living animal preserves ALG-1:RNA binding context, and (3) viable *alg-1* genetic mutants exist to use as specificity controls. These advantages, along with those of iCLIP, allow us to identify miRNA:mRNA interaction sites in an entire organism with increased sensitivity and resolution.

2. iCLIP Protocol

2.1 Materials

2.1.1 Buffers—

Buffer	Composition
M9 Buffer	22mM KH ₂ PO ₄ ; 22mM Na ₂ HPO ₄ ; 85mM NaCl; 1mM MgSO ₄
Homogenization Buffer	100mM NaCl; 25mM HEPES, pH 7.5; 250μM EDTA, pH 8.0; 0.1% (w/v) NP-40; 2mM DTT; 25U/mL rRNasin (Promega); 1 tablet / 20mL Complete Mini Protease Inhibitor (Roche)
RNase I Dilution Buffer	10mM Tris-HCl, pH 8.0; 100mM NaCl; 50% Glycerol
High Salt Wash Buffer	50mM Tris-HCl, pH 7.4; 1M NaCl; 1mM EDTA; 1% NP-40; 0.1% SDS; 0.5% sodium deoxycholate
Wash Buffer	20mM Tris-HCl, pH 7.4; 10mM MgCl ₂ ; 0.2% Tween-20
5X PNK Buffer, pH 6.5	350mM Tris-HCl, pH 6.5; 50mM MgCl ₂ ; 25mM DTT
4X Ligation Buffer	200 mM Tris-HCl, pH 7.4; 40mM MgCl ₂ ; 40mM DTT
MOPS-SDS Running Buffer	50mM MOPS; 50mM Tris-base; 1mM EDTA; 0.1% SDS
Bis-Tris Supplemented Transfer Buffer	25mM Bicine; 25mM Bis-Tris; 1mM EDTA; 5% Methanol; 0.01% SDS
PK Buffer	100mM Tris-HCl, pH 7.4; 50mM NaCl; 10mM EDTA
7M Urea / PK Buffer (must be made fresh)	100mM Tris-HCl, pH 7.4; 50mM NaCl; 10mM EDTA; 7M urea
Gel Extraction Buffer	300mM NaCl; 10mM Tris-HCl, pH 8.5
2X TBE-Urea Sample Buffer	90mM Tris-Base; 90mM Boric acid; 2mM EDTA; 12% Ficoll Type 400; 7M Urea; 0.01% Bromophenol Blue; 0.05% Xylene Cyanol

2.1.2 Antibodies

2.1.3 Enzymes and other material—

Material	Supplier
Protein G Dynabeads	Life Technologies
-ALG-1 antibody (PA1-031)	Thermo Scientific (Pierce Antibodies)
Turbo DNase	Ambion
RNase I	Ambion
PNK Enzyme	NEB
rRNasin	Promega
T4 RNA Ligase I	NEB
PEG400	Sigma Aldrich

Material	Supplier
³² P- ATP	PerkinElmer
4x NuPAGE LDS Sample Buffer	Life Technologies
4–12% Bis-Tris NuPAGE Gel	Life Technologies
NuPAGE Antioxidant	Life Technologies
Pure nitrocellulose membrane (0.45µm pore size)	Bio-Rad
Proteinase K (fungal)	Roche
Acid Phenol / Chloroform (5:1)	Ambion
Chloroform:IAA (25:1)	Sigma Aldrich
UreaGel System	National Diagnostics
Dark Reader Transilluminator	Clare Chemical Research
SYBR-Gold	Life Technologies
0.45µm Costar-X spin column	Corning
CircLigase II	Epicentre
BamHI	NEB
Accuprime Supermix I	Life Technologies
6% TBE gel	Life Technologies

2.1.4 Oligonucleotides

L3, 100nmol HPLC Purified, IDT:

/5rApp/AGATCGGAAGAGCGGTTCAG/3ddc/

**Rclip Reverse Transcriptase Primers with Different Bar Codes:
Standard Desalting IDT**

Rclip: /5phos/NNXXXXNNNAGATCGGAAGAGCGTCTGgataCTGAACCGC

XXXX = A unique 4 nucleotide sequence (i.e. ATCG)

Cut_oligo_ddc:

GTTTCAGGATCCACGACGCTCTTC/ddc/

P5Solexa (Paired End PCR Primer 1.0 - Illumina), PAGE Purified, IDT:

AATGATACGGGACCAACGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCT

P3Solexa (Paired End PCR Primer 2.0 - Illumina), PAGE Purified, IDT:

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT

lin-41 LCS Primer 1:

GAGGCAGAATGGTTGTATAA

lin-41 LCS Primer 2:

TTATACAACCGTTCTACACTCA

lin-41 Control Primer 1:

ACATGTTTCTGGGCGATAGG

lin-41 Control Primer 2:

CGTGCTGTTGGCTACTTCAA

2.2 Procedure

2.2.1 Growing worms and preparing lysates

- 1 Grow worms as desired so that at least 50,000 worms or enough to obtain ~1mg of total protein per sample.
- 2 Wash worms (3x) with 10mL M9 in 15mL tube. Resuspend in 5mL M9 after washes.
- 3 Rock worms for 20min on nutator at temperature used for culturing of samples. Spin down worms in clinical centrifuge for 30 seconds.
- 4 Plate worms on Nematode Growth Medium (NGM) plates with no food at room temperature. Be sure to avoid areas of high concentration and overlapping worms.
- 5 Place the NGM plates with worms into Spectrolinker XL-1000 (without the plate lids) and irradiate with UV-B at 3kJ/m^2 (Energy Settings: 3000). Check worms using microscope to be sure all worms have been completely immobilized. Collect worms in M9 buffer and transfer to 2mL round bottom microcentrifuge tube. Spin worms for 30 seconds in desktop centrifuge.
- 6 Remove excess M9 buffer and resuspend the worms in 700 μL of ice-cold homogenization buffer. Keep worms on ice.
- 7 Sonicate the worms (5x) with 10 second pulses and 50 seconds resting on ice between pulses (~18W RMS output power on Dismembrator Model 100, Fisher Scientific).
- 10 Spin lysates at $16000\times g$ for 15 minutes at 4°C . Transfer supernatant to new 1.5mL microcentrifuge tube.
- 11 Freeze lysates in dry-ice-ethanol bath. Store at -80°C .

2.2.2 RNA trimming and immunoprecipitation

- 1 Thaw lysates at 4°C on nutator for 30 minutes.
- 2 Quantify the protein content of lysate samples using bradford assay. Adjust lysate concentrations to be equal between all samples (~1–3mg/mL) with ice-cold homogenization buffer.
- 3 Save 20 μL of sample for western blot analysis (input).
- 4 While quantifying, aliquot 50 μL Protein G Dynabeads per sample. Wash beads (2x) with homogenization buffer.
- 5 Resuspend beads in 100 μL of homogenization buffer.
- 6 Preclear lysates by adding 100 μL 50:50 bead slurry and rocking on nutator for 1 hour at 4°C .
- 7 Pellet beads+lysates using magnetic rack. Transfer lysate to new chilled 1.5mL microcentrifuge tube. Keep samples on ice.
- 8 Add 2 μL TurboDNase and 10 μL RNase I dilution (1/500 RNase I dilutions are used for library preparation, while 1/50 dilutions are used to control for antibody specificity).
- 9 Incubate samples for 3min in Thermomixer R set to 37°C and shaking at 1100rpm. Immediately transfer samples to ice.

- 10 Add 7µg -ALG-1 antibody to lysate. Rock lysate+antibody at 4°C overnight.
- 9 Aliquot 100µL Protein G Dynabeads per sample. Wash beads (2x) with homogenization buffer.
- 10 Add beads to lysate+antibody. Incubate at 4°C for 1 hour.
- 11 Collect the beads with magnetic rack.
- 12 Save 20µL of supernatant for western blot analysis (supernatant).
- 13 Wash (2x) the beads with 900µL ice-cold high salt wash buffer.
- 14 Wash (2x) the beads with 900µL ice-cold wash buffer.

2.2.3 Dephosphorylation of RNA 3' ends

1. Collect the beads with magnetic rack. Discard supernatant and resuspend beads in 20µL dephosphoylation mix (15µL ddH₂O; 4µL 5X PNK, pH 6.5 buffer; 0.5µL PNK enzyme; 0.5µL rRNasin).
2. Incubate for 20 minutes at 37°C.
3. Add 500µL wash buffer.
4. Wash (1x) with 900µL high-salt buffer.
5. Wash (2x) with 900µL wash buffer.

2.2.4 Linker ligation to RNA 3' end

1. Collect the beads with magnetic rack. Discard supernatant and resuspend beads in 16µL ligation mix (9µL ddH₂O; 4µL 4X ligation buffer; 1µL T4 ssRNA Ligase 1; 0.5µL rRNasin; 1.5µL 20µM L3 oligo).
2. Add 4µL PEG(400) to each reaction.
3. Incubate overnight in Thermomixer R at 16°C with intermittent shaking: 1300rpm for 15 seconds every 5min.
4. Add 500µL wash buffer.
5. Wash (2x) with 900µL high salt wash buffer.
6. Wash (2x) with 900µL wash buffer.

2.2.5 End labeling of RNA 5' end

1. Remove the supernatant and resuspend the beads in 80µL of hot PNK mix (67µL ddH₂O; 8µL 10X PNK buffer; 1µL P³²- -ATP; 4µL T4 PNK enzyme).
2. Incubate for 10 minutes in Thermomixer R at 37°C with intermittent shaking: 1200rpm for 15 seconds every 4min.
3. Add 500µL wash buffer.
4. Collect beads with magnetic rack and remove supernatant. Dispose of liquid as radioactive waste.
5. Resuspend the beads in 30µL wash buffer and 10µL 4x NuPAGE LDS sample buffer. Add 2µL 1M DTT.
6. Incubate in Thermomixer R at 70°C for 10 minutes at 1200rpm.
7. Immediately place samples on magnetic rack.

2.2.6 SDS-PAGE and nitrocellulose transfer

1. Load the samples on a 4–12% Bis-Tris gel with 1X MOPS-SDS running buffer. Before running add 500 μ L NuPAGE Antioxidant to upper chamber of running buffer. Run gel at 180V.
2. Transfer the RNA-protein complexes from the gel to a pure nitrocellulose membrane (0.45 μ m pore size) with Bis-Tris supplemented transfer buffer at 40V for 4 hours at 4°C.
3. After the transfer, rinse the membrane in 1X PBS buffer. Wrap membrane in saran wrap and expose it to autoradiography film. Perform 1 hour, 2 hour, and overnight exposures. See Figure 2.
4. Using an exposed piece of film as a mask, cut out the band corresponding to the ALG-1/RNA complexes in the low RNase condition with a clean razor blade. Cut isolated membrane into smaller pieces and place into a 1.5mL microcentrifuge tube. Pieces of membrane can be stored at –80C.

2.2.7 RNA isolation of purification

1. Prepare 4mg/mL Proteinase K solution in 1X PK buffer and incubate this solution at 37°C for 20 minutes to deactivate any RNases that may be present.
2. Add 200 μ L of prepared Proteinase K solution to each tube of isolated nitrocellulose pieces and incubate for 20 minutes at 37°C at 1200rpm.
3. Add 200 μ L of 7M urea / 1X PK Buffer and incubate for 20 minutes at 37°C at 1200rpm.
4. Add 500 μ L of Acid Phenol/Chloroform. Briefly shake tubes by hand. Incubate at 37°C for 20 minutes at 37°C at 1200rpm.
5. Mix tube vigorously by hand for a few seconds and centrifuge the tubes at 16000 $\times g$ for 5 minutes at room temperature.
6. Transfer aqueous layer to new 1.5mL microcentrifuge tube. Add 400 μ L chloroform:IAA. Mix briefly by hand and centrifuge the tubes at 16000 $\times g$ for 5 minutes at room temperature.
7. Transfer the aqueous layer to a new 1.5mL microcentrifuge tube and add 40 μ L 3M sodium acetate, pH 5.2; 1 μ L 20mg/mL glycogen; 1mL 1:1 ethanol:isopropanol. Mix.
8. Precipitate overnight at –20°C.

2.2.8 Reverse transcription

1. Spin for 20 minutes at 16000 $\times g$ at 4°C.
2. Remove the supernatant and wash the pellet with 500 μ L 80% ethanol.
3. Air dry pellet for exactly 8 minutes.
4. Resuspend the pellet in 7.25 μ L RNA/primer mix (use different Rclip primer for each experiment/replicate): 6.25 μ L ddH₂O; 0.5 μ L 0.5 μ M Rclip primer; 0.5 μ L 10mM dNTP.
5. Incubate for 5 minutes at 70°C before cooling to 25°C.
6. Add 2.75 μ L RT mix: 2 μ L 5X First-Strand RT buffer; 0.5 μ L 0.1M DTT; 0.25 μ L Superscript III.

7. Incubate 5 minutes at 25°C, 20 minutes at 42°C, 40 minutes at 50°C, and 5 minutes at 80°C before cooling to 4°C.
8. Add the following: 90µL TE buffer; 10µL 3M sodium acetate, pH 5.2; 1µL 20mg/mL glycogen; 250µL 100% ethanol. Mix.
9. Precipitate overnight at -20°C.

2.2.9 Gel purification of cDNA

- 1 Pour 6% TBE-urea gel using UreaGel System (2.4mL concentrate, 6.6mL diluent, 1mL system buffer, 90µL 10% APS, 4µL TEMED).
- 2 Allow gel to polymerize for at least 1 hour.
- 3 Pre-run gel with 1X TBE buffer at 150V for 30min.
- 4 Spin for 20 minutes at 16000 $\times$ g at 4°C.
- 5 Remove the supernatant and wash the pellet with 500µL 80% ethanol.
- 6 Air dry pellet for exactly 8 minutes.
- 7 Resuspend pellet in 10µL ddH₂O and add 10µL 2x TBE-urea loading buffer. Heat samples to 80°C for 3 minutes directly before loading.
- 8 Load samples and low molecular weight marker on prepared 6% TBE-Urea gel and run at 180V.
- 9 Remove gel from glass plates and stain with SYBR-GOLD for 10 minutes.
- 10 Prepare 3 0.5mL microcentrifuge tubes for each sample by poking 3–4 holes in the bottom of each tube with 21.5 gauge needle.
- 10 Using dark-reader, cut three sections of gel from each lane that correspond to the sizes: 200-120nt (high), 120-85nt (medium), and 85-70nt (low). See Figure 3.
- 11 Place each gel slice into prepared 0.5mL microcentrifuge tubes.
- 12 Centrifuge gel pieces at 16000 $\times$ g for 2 minutes to fragment the gel slices.
- 13 Add 400µL gel extraction buffer.
- 14 Rock on nutator for 2 days at 4°C; alternatively, rock on nutator for 2 hours at 37°C.
- 15 Transfer supernatant and gel pieces to 0.45µm filter column and spin at 600 $\times$ g for 10 seconds.
- 16 To flow-through add: 40µL 3M sodium acetate, pH 5.2; 1µL 20mg/mL glycogen; 1mL 100% ethanol. Mix.
- 17 Precipitate overnight at -20°C.

2.2.10 Circularization and linearization of cDNA

1. Spin for 20 minutes at 16000 $\times$ g at 4°C.
2. Remove the supernatant and wash the pellet with 500µL 80% ethanol.
3. Air dry pellet for exactly 8 minutes.
4. Resuspend in 8µL ssDNA ligation mix: 6.5µL ddH₂O; 0.8µL 10X CircLigase II buffer; 0.4µL 50mM MnCl₂; 0.3 CircLigase II enzyme.

5. Incubate at 60°C for 1 hour.
6. Heat inactivate enzyme by incubating for 15 minutes at 80°C.
7. Add 30µL oligo annealing mix: 23µL ddH₂O; 3µL 10X NEBuffer 3; 3µL 10X BSA; 1µL 10µM Cut_oligo_ddc.
8. Incubate for 1 minute at 95°C for 1 minute, then decrease the temperature every 20 seconds by 1°C until 25°C has been reached. (This can be accomplished using the Thermomixer R and changing the temperature from 95°C to 25°C, which will take approximately 25 minutes to adjust.)
9. Add 2µL BamHI and incubate for 30 minutes at 37°C.
10. Add the following: 50µL TE buffer; 10µL 3M sodium acetate, pH 5.2; 1µL 20mg/mL glycogen; 300µL 100% ethanol.
11. Precipitate overnight at -20°C.

2.2.11 PCR Amplification

1. Spin for 20 minutes at 16000 $\times$ g at 4°C.
2. Remove the supernatant and wash the pellet with 500µL 80% ethanol.
3. Air dry pellet for exactly 8 minutes.
4. Resuspend pellet in 19µL ddH₂O and transfer to a PCR tube.
5. To the cDNA, add 1µL P5/P3 Solexa Mix (10µM each) and 20µL Accuprime Supermix I.
6. Run the following PCR Program: 94°C – 2min, [94°C – 15sec, 65°C – 30sec, 68°C – 30sec] 25–35 cycles, 68°C – 3min, 4°C hold.
7. Mix 8µL PCR product with 2µL 5xTBE loading buffer and load on a precast 6% TBE gel. Stain the gel with SYBR-GOLD or ethidium-bromide and image. See Figure 4A.

2.2.12 Quantification and sequencing

1. Quantify library concentration using QPCR and bioanalyzer.
2. Submit 15µL of the library for multiplex sequencing and store the rest at -20°C.

2.2.13 PCR Control for ALG-1 binding specificity

1. Prepare PCR reaction (1µL iCLIP library, 17.25µL ddH₂O; 5µL 5X Go-Taq Buffer; 1µL 25mM MgCl₂; 0.5µL 10mM dNTPs; 0.5µL *lin-41* LCS Primer 1+2 Mix (10µM each); 0.25µL Go-Taq polymerase).
2. Run the following PCR program: 94°C – 2min, [94°C – 15sec, 48°C – 30sec, 72°C – 30sec] 38 cycles, 72°C – 3min, 4°C hold. When using *lin-41* Control Primers 1+2, anneal at 55°C.
3. Run 10µL PCR product on 2% agarose gel and stain with ethidium-bromide. Expected product size of *lin-41* LCS 1+2 is 60 nts. See Figure 4B.

3. Notes

We have made several modifications to the iCLIP protocol [19]. First, consistent with our previously published protocol for ALG-1 CLIP-seq [22], we preclear lysates before RNA trimming and immunoprecipitation. This reduces background seen on the autoradiograph

from non-specific interaction with the beads. If using another antibody, we recommend that users thoroughly evaluate immunoprecipitation conditions and specificity of the antibody before proceeding with iCLIP. To further improve the autoradiograph, we prepare RNA-protein samples under reducing conditions to prevent the antibody complex from interfering with the ALG-1 signal. As can be seen in Figure 2, the difference between high and low RNase conditions is not as striking as seen with smaller proteins. For assessing immunoprecipitation efficiency, we recommend saving protein samples during the immunoprecipitation and after ligation of the 3' adapter for subsequent western blot analysis. To increase recovery of cDNA from denaturing PAGE, we prefer an extraction time of 2 days at 4°C, as opposed to 2 hours at 37°C. In some cases, we had difficulty with ligation of the adapter used to generate dsDNA for restriction enzyme digest (Cut_oligo) to the reverse transcription primer (Rclip). This generated a distinct band (112 nts) in our evaluation of the cDNA library (Figure 4A), which accounted for ~90% of the PCR amplified library in some cases. To address this issue, we added a heat inactivation step after circularization and designed an adapter that has 3' dideoxy cytosine instead of 4 adenosines. These additions eliminated these non-specific products and led to cleaner libraries (Figure 4A). As an assessment of the quality of the library, we recommend an additional PCR step to detect the sequences known to be bound by the miRNA complex in the sample (e.g. the let-7 complementary site in the *lin-41* 3' UTR for extracts from L4 stage worms), as this provides evidence of the sensitivity and specificity of the library (Figure 4B). As an additional control, we also recommend using primers that amplify an exon of *lin-41* that is not known to be bound by ALG-1.

4. Conclusions

The use of CLIP-based approaches to identify miRNA target sites has furthered the understanding of how these small RNAs are able to interact with their target mRNAs [12], revealed a novel miRNA interaction with a non-coding RNA [7], and provided comprehensive lists of Ago/miRNA-mediated interactions [4–6,9–11]. Here we have brought an advancement on the original CLIP methodology, iCLIP, to *C. elegans*, a powerful genetic model. The increased recovery of transcripts, as well as the ability to identify crosslink sites through mutations and the truncated cDNAs, allows iCLIP to be significantly more sensitive than other CLIP-based methods [17]. Improvements such as iCLIP and other future innovations to CLIP-based approaches have the potential to generate comprehensive lists of Ago/miRNA targets in a variety of specific cells, tissues, and genetic backgrounds that will increase our understanding of miRNA function.

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Highlights

Determining endogenous miRNA target sites has remained a challenge

CLIP-based methods enable the identification of endogenous miRNA targets

iCLIP provides increased sensitivity and resolution by isolating all cDNA products

We have adapted iCLIP for the isolation of ALG-1 binding sites in *C. elegans*

Fig. 1.

Outline of the ALG-1 iCLIP method: i) *in vivo* crosslinking stabilizes RNA-protein interactions and lysates are generated by sonication of immobilized animals; ii) lysates and beads are prepared for digestion and immunoprecipitation; iii) RNA molecules are trimmed by RNase I; iv) ALG-1 and crosslinked RNAs are immunoprecipitated using specific antibody and stringent wash conditions; v) the 2' 3' cyclic monophosphate generated by RNase I is removed by Polynucleotide Kinase in low pH (6.5) conditions; vi) ligation of adapter oligo to the 3' hydroxyl group of the RNA fragments (circularization and concatemer formation events are prevented by the presence of 3' dideoxy cytosine on the adapter); vii) the 5' end of the RNA is radiolabeled by Polynucleotide Kinase using ^{32}P -ATP; viii) samples are analyzed by electrophoresis on native SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane to isolate the band corresponding to ALG-1/RNA complexes; ix) ALG-1 is removed from the RNA by Proteinase K digestion and the RNA is purified; x) reverse transcription of isolated RNA using phosphorylated oligo; xi) cDNA is separated by size on denaturing PAGE and purified from the gel to remove sequences that are too long or consist of only adapter and oligo; xii) purified cDNA is circularized, an adapter is added that generates a specific dsDNA region in the sequence of the reverse transcription primer, and is linearized by restriction enzyme digest; xiii) PCR amplification; xiv) evaluation of library by complexity, size, and concentration; xv) high-throughput sequencing using the Illumina GAIIX system.

Fig 2.

Autoradiograph of protein cross-linked to RNA fragments. Black arrow indicates expected migration size of ALG-1. Red square denotes region of membrane that was excised for RNA isolation and purification. RNA was partially digested by RNase I in either high (++) or low (+) concentrations. High (++) RNase treatments serve as a control for antibody specificity, as the band should be less diffuse than that of low RNase. The ALG-1 signal is absent in the no-antibody control. The bands seen below the ALG-1 signal (~52 and 38kDa) are non-specific as they are observed in the no-antibody lane in longer exposures, while the signal at ~150kDa is not.

Fig. 3.

Representation of denaturing PAGE used for isolating cDNA. The gel is stained with SYBR-gold and viewed using a Dark Reader. Note that only the ladder and Rclip primer will be visible at this point, as the cDNA is not in high enough concentrations to be detected. Using a clean razor blade, the lanes corresponding to each sample are removed (red lines). The lanes are divided into high (H), medium (M), and low (L) fractions. Avoid cutting below the region of 70 nts, as the presence of adapter-Rclip primer product (52 nts) can lead to libraries that are not sufficiently complex for analysis. XCFF, Xylene cyanol FF; BPB, Bromophenol blue.

Fig. 4.

(A) Comparison of cDNA libraries from the original iCLIP protocol and the modified iCLIP protocol presented here by non-denaturing PAGE. Diffuse bands corresponding to the range of sizes for each cDNA fraction removed from denaturing PAGE with 76 nts of extension by PCR primers should be observed. The expected migration size of the primer-dimer (128 nts) that contains no sequence information, the aberrant ligation product (112 nts), and the primers (58 nts and 61 nts) are indicated by black arrows. The primer-dimer product should not be present in libraries submitted for sequencing. A band corresponding to miRNA only product (~150nt) may be seen in some samples. (B) PCR controls for cDNA library specificity. The *let-7* complementary site (LCS) in the 3'UTR of *lin-41* is a well characterized ALG-1 interaction site and is expected to be present in iCLIP cDNA libraries. PCR of this region is expected to produce a product, while PCR of exonic regions of *lin-41*, that are not known to be bound by ALG-1, should not produce a product. Control PCRs for WT genomic DNA (gDNA) are shown.