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Optimizing lncRNA-miRNA interaction analysis: Modified crosslinking and immunoprecipitation (M-CLIP) assay ☆

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ABSTRACT

Defining lncRNA-miRNA interactions is critical for understanding their roles in cellular signaling and cancer biology. Capturing these interactions is challenging due to the inherent instability of RNAs. Our study focuses on the long non-coding RNA (lncRNA) UCA1, exploring its role in ovarian cancer progression through interactions with microRNAs (miRNAs). We hypothesized that UCA1 acts as a competing endogenous RNA (ceRNA), sequestering let-7 miRNAs to modulate the expression of let-7 targets, thereby driving cancer progression. Typically, miRNAs associate with ribonucleoprotein complexes that include Ago2 protein, pivotal in mediating miRNA activity and stability. Analyzing these complexes has proven effective in identifying lncRNAs and their miRNA partners. Inspired by previous RNA-protein crosslinking methodologies, we developed the Modified Crosslinking and Immunoprecipitation (M-CLIP) assay to capture UCA1-let-7 miRNA interactions through immunoprecipitation of Ago2, followed by qRT-PCR to detect the bound UCA1 and its associated let-7 miRNAs. This method includes:

- Formaldehyde-based crosslinking followed by cell lysis
- Immunoprecipitation and isolation of RNAs bound to bait proteins
- Characterization of bound lncRNA and target miRNAs

Our findings demonstrate the efficacy of the M-CLIP assay in identifying UCA1-let-7 interactions, providing a robust tool to elucidate how UCA1 and similar lncRNAs influence cancer progression through miRNA sequestration.

☆ **Related research article:** Ha JH, Radhakrishnan R, Nadhan R, Gomathinayagam R, Jayaraman M, Yan M, Kashyap S, Fung KM, Xu C, Bhattacharya R, Mukherjee P, Isidoro C, Song YS, Dhanasekaran DN. Deciphering a GPCR-lncrna-miRNA nexus: Identification of an aberrant therapeutic target in ovarian cancer. Cancer Lett. 2024 Jun 1;591:216,891. doi: 10.1016/j.canlet.2024.216891. Epub 2024 Apr 18. PMID: 38,642,607.

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Specifications Table

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Background

Long non-coding RNAs (lncRNAs) play crucial roles in regulating various biological processes, including cellular differentiation, proliferation, and apoptosis [1,2]. In cancer biology, their interaction with microRNAs (miRNAs) as competing endogenous RNAs (ceRNAs) is particularly important [3]. These interactions sequester miRNAs, preventing them from binding to mRNA targets, thus altering gene expression patterns critical for cancer progression and metastasis [4]. Understanding these interactions is essential for elucidating the complex regulatory networks that contribute to disease pathology [2].

Despite the recognized importance of lncRNA-miRNA interactions, traditional methodologies for studying RNA-protein interactions, such as Crosslinking and Immunoprecipitation (CLIP) assays, are not optimized for detecting these interactions. Conventional CLIP assays involve crosslinking of RNA to protein by UV or chemical means, followed by immunoprecipitating the RNA-protein complex to analyze the RNA component and infer functional and regulatory mechanisms [5]. While effective for studying RNA-protein interactions, these methods fall short in specifically targeting lncRNA-miRNA interactions, which are vital for understanding the broader functional roles of lncRNAs in cancer and other diseases.

To address this limitation, researchers have modified the CLIP methodology to focus on lncRNA-miRNA interactions within ribonucleoprotein complexes involving Argonaute 2 (Ago2), a key protein component of the RNA-induced silencing complex (RISC) that mediates miRNA activity and stability [6–8]. By incorporating Ago2 antibodies into the immunoprecipitation protocol, one can selectively analyze Ago2-immunecomplex containing both lncRNAs and the miRNAs they sequester [9]. However, the detailed procedural steps of these adaptations have often been underreported, leaving gaps in reproducibility and standardization.

Our work builds on these initial adaptations by providing a comprehensive, step-by-step procedure for the Modified Crosslinking and Immunoprecipitation (M-CLIP) assay, specifically optimized for in vitro analysis of lncRNA-miRNA interactions. We have refined and optimized the previously described CLIP assay methodology [5] to include detailed descriptions of each stage including formaldehyde-based crosslinking of RNA-protein complexes in situ, cellular lysis, and protein isolation, immunoprecipitation using Ago2/control IgG antibodies, reversal of crosslinking, RNA isolation, and quantitative real time polymerase chain reaction (qRT-PCR) for the lncRNA UCA1 and its target miRNAs (let-7 miRNAs) in OVCAR8, a high-grade serous ovarian carcinoma (HGSOC) cell line.

These enhancements significantly improve the assay's specificity, efficiency, and reproducibility, addressing previous gaps in the methodology. By providing a robust tool for probing the intricate molecular mechanisms underlying lncRNA function, our M-CLIP assay facilitates a deeper understanding of how lncRNA-miRNA interactions contribute to cancer progression and other complex diseases. This detailed methodological framework will enable researchers to more accurately and reliably study these critical interactions, advancing our knowledge in the field and potentially informing new therapeutic strategies.

Moreover, the applicability of the M-CLIP assay extends beyond cancer research, offering potential insights into various biological contexts where lncRNAs and miRNAs play crucial regulatory roles. By standardizing and optimizing this methodology, we aim to make it accessible for a broader range of studies, fostering innovation and discovery in RNA biology.

Method details

The whole method has been elaborated in a stepwise manner as indicated in the Flowchart (Fig. 1). Table 1 includes a detail list of reagents used for the M-CLIP assay.

Cell Culture

- **Materials Needed**:

Tissue Culture Biosafety Cabinet
Tissue Culture Incubator
HGSOC cell line, OVCAR8
RPMI-1640 cell culture media supplemented with 10 % FBS (Fetal Bovine Serum)
50 U/mL penicillin and 50 µg/mL streptomycin
100 mm cell culture plates
Trypsin
Sterile IX Phosphate Buffered Saline (PBS, 3.2 mM NaH2PO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.4)
Centrifuge
Pipettes

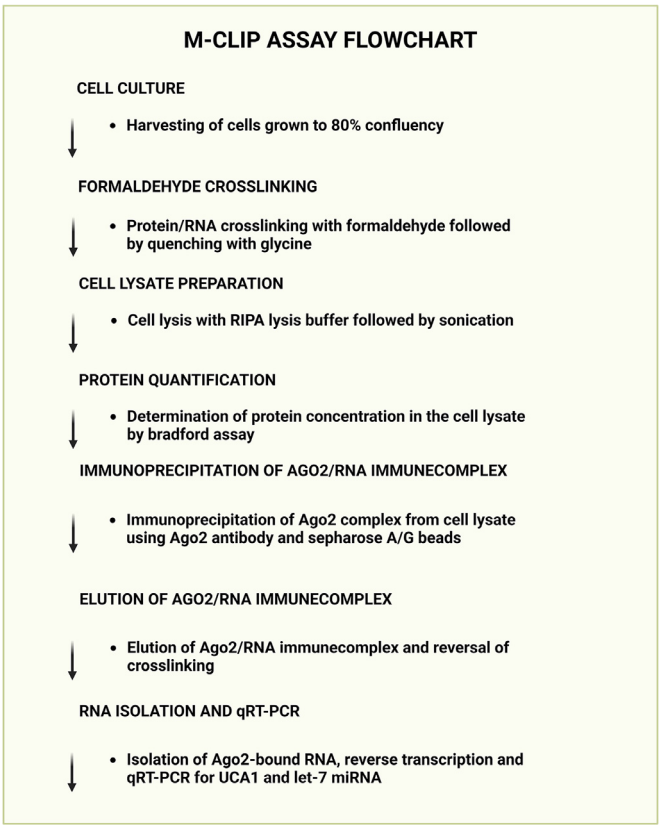

Fig. 1. Workflow of the Modified Crosslinking and Immunoprecipitation (M-CLIP) Assay This flowchart summarizes the key steps of the M-CLIP assay for analyzing lncRNA-miRNA interactions. Cells are harvested and crosslinked using formaldehyde, followed by lysis and immunoprecipitation of Ago2-bound complexes. Crosslinking is reversed, and RNA is isolated and analyzed by qRT-PCR to detect target lncRNA-miRNA interactions, specifically UCA1-let-7 in this study.

15 ml centrifuge tubes

• **Procedure**:

1. Seed OVCAR8 cells at a density of 1.5×10^6 cells/100 mm cell culture plate in 10 ml of RPMI-1640 media supplemented with 10 % FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin.
2. Maintain cell cultures at 37 ˚C in a 5 % CO2 incubator.
3. Allow cells to grow for 48 h until they reach 80 % confluency.
4. Trypsinize cells and pool from five cell culture plates into a 15 ml centrifuge tube.
5. Centrifuge the cell suspension at 1200 rpm for 3 min at room temperature in a tabletop multipurpose centrifuge.
6. Discard the supernatant and resuspend the cell pellet in 10 mL of sterile 1X PBS.
7. Centrifuge the cell suspension at 1200 rpm for 3 min at room temperature and discard the supernatant.
8. Repeat the PBS wash step twice.
9. Resuspend the cell pellet in 10 mL of sterile 1X PBS for formaldehyde crosslinking.

2. **Formaldehyde Crosslinking**

• **Materials Needed**:

37 % formaldehyde
2.5 M glycine (pH 7.0)
Ice cold 1X PBS
Centrifuge
Pipettes
Mini Tube Rotator pH Meter
Laboratory Precision Weighing Balance

• **Procedure**:

Table 1
List of Reagents.

Product	Vendor	Catalogue Number
Acetic Acid	Macron Chemicals, USA	V193–45
Agarose	Fisher Scientific, NH, USA	BP160–500
Ago2 Antibody	Active Motif, CA, USA	39,583
Bio-Rad iScript cDNA synthesis kit	Bio-Rad, CA, USA	1,708,891
Bradford Reagent	Bio-Rad, CA, USA	5,000,006
Dithiothreitol (DTT)	Research Organics Inc, OH, USA	2190D
DNA Ladder – 100 bp	Promega, WI, USA	G2101
DNA Ladder – 1 kb	Promega, WI, USA	G5711
Ethidium Bromide Solution (10mg/ml)	Bio-Rad, CA, USA	1,610,433
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, MO, USA	E5134
Fetal Bovine Serum (FBS)	Gibco, USA	10,099,141
Formaldehyde solution (37 %)	Sigma Aldrich, MO, USA	F8775
Glycine	J T Baker, NJ, USA	405,906
Go Taq Green PCR Master Mix	Promega, WI, USA	M712B
IgG Mouse Antibody	Santa Cruz Biotechnology, TX, USA	sc-2025
miScript SYBR Green PCR kit	Qiagen, MD, USA	218,076
Nuclease Free Water	Qiagen, MD, USA	129,115
Penicillin-Streptomycin Solution (50X)	Corning, NY, USA	30001CI
Phosphate Buffered Saline (PBS) (1X)	Corning, NY, USA	MT21040CM
Protease/ Phosphatase Inhibitor Cocktail (100x)	Cell Signaling Technologies, MA, USA	5872
Protein Sepharose A Beads (eZview Red Protein A Affinity Gel)	Sigma Aldrich, MO, USA	P6486
Protein Sepharose G Beads (eZview Red Protein G Affinity Gel)	Sigma Aldrich, MO, USA	E3403
miScript II RT kit	Qiagen, MD, USA	218,161
RNeasy Plus Mini Kit	Qiagen, MD, USA	74,136
RIPA Buffer (10x)	Cell Signaling Technologies, MA, USA	9806S
RPMI 1640 with L-Glutamine	Corning, NY, USA	10040CM
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, MO, USA	L4390
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad, CA, USA	1,725,274
TRIS	Sigma Aldrich, MO, USA	T-1503
TRIS-Cl	J T Baker, NJ, USA	4103–02
Trypsin 0.25 %	Corning, NY, USA	25052CI

1. Add 270 µL of 37 % formaldehyde to the cells suspended in 10 mL of 1X PBS (final concentration of 1 % v/v, 0.36 M) to enable RNA-protein crosslinking.
2. Incubate the suspension for 10 min at room temperature with slow rotation in a tube rotator.
3. Add 1 mL of 2.5 M glycine (pH 7.0) to quench the crosslinking (final concentration of 0.25 M glycine).
4. Incubate for 5 min at room temperature with slow rotation in a tube rotator.
5. Harvest cells by centrifuging at 3000 rpm for 4 min at room temperature.
6. Resuspend cell pellets in 10 mL of ice-cold 1X PBS and centrifuge at 1200 rpm for 3 min at room temperature.
7. Repeat the PBS wash step once more and discard the supernatant.
8. Proceed to protein isolation.

3. Preparation of Cell Lysate

- **Materials Needed**:

1X Radio Immunoprecipitation Assay (RIPA) Lysis buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 % NP40, 1 % (w/v) Sodium Deoxycholate, 2.5 mM Sodium Pyrophosphate, 1 mM Glycerophosphate, 1 mM Sodium Orthovanadate, and 1 mg/ml leupeptin)

Phosphatase/Protease Inhibitor Cocktail (Proprietary mix of Aprotinin, Bestatin, E64, Leupeptin, sodium fluoride, sodium pyrophosphate, β-glycerophosphate, and sodium orthovanadate)

Sonicator (Branson Digital Sonifier 450 with microtip)

Centrifuge

Vortex Lab Mixer

1.5 mL microcentrifuge tube

Pipettes

Procedure:

1. Resuspend the cell pellet in 1 mL of 1X RIPA buffer and 10 µL of Phosphatase/Protease Inhibitor Cocktail, and transfer to a 1.5 mL microcentrifuge tube.
2. Incubate the cell suspension on ice for 15 min, vortexing every 5 min.
3. Sonicate the cell suspension for 12 s at 10 % amplitude, repeating this three times with 2 min incubation on ice between each cycle.

4. Centrifuge the cell lysate at 14,000 rpm for 10 min at 4 ˚C.
5. Collect the supernatant containing the cellular proteins with bound RNAs.

4. Protein Quantification

- **Materials Needed**:

1X RIPA Lysis Buffer
Nuclease-free water
Bradford reagent
Nanophotometer
Procedure:

1. Add 799 μL of nuclease-free water, 200 μL of Bradford reagent, and 1 μL of cell lysate sample to a 1.5 mL microcentrifuge tube.
2. For the control tube, add 1 μL of 1X RIPA buffer instead of the cell lysate sample.
3. Mix by inverting the tubes several times and incubate at room temperature (25 ˚C) for 5 min.
4. Measure protein concentration using a Nanophotometer against the control sample, at a wavelength of 595nm.

5. Immunoprecipitation Using Ago2/Control IgG Antibodies

- **Materials Needed**:

Ago2 antibody
IgG rabbit antibody (control)
Protein Sepharose A and G beads
1X RIPA buffer
Phosphatase/Protease Inhibitor Cocktail
Mini Tube Rotator
Vortex Lab Mixer
Centrifuge
1.5 mL microcentrifuge tube
Pipettes
Procedure:

1. Quantify the protein in the cell lysate and transfer the volume with 2 mg of protein to two 1.5 mL microcentrifuge tubes.
2. Add 10 μL of Ago2 antibody to one tube and 10 μL of IgG rabbit antibody to the other tube as a control.
3. Vortex the tubes and incubate at 4 ˚C for 2 h with slow mixing in a tube rotator.
4. Meanwhile, aliquot 25 μL each of protein Sepharose A and G beads into two 1.5 mL microcentrifuge tubes.
5. Wash the beads twice with 1X RIPA buffer containing Phosphatase/Protease Inhibitor Cocktail, vortexing and centrifuging at 10,000 rpm for 30 s at 4 ˚C.
6. After the 2-hour incubation, centrifuge the tubes briefly and add the cell lysate-antibody suspension to the tubes with activated beads.
7. Vortex the tubes and incubate at 4 ˚C for 2 h with slow mixing in a tube rotator.

6. Reversal **of Crosslinking and RNA Isolation**

- **Materials Needed**:

Elution buffer (50 mM Tris–Cl, pH 7.0, 5 mM EDTA, 10 mM dithiothreitol (DTT) and 1 % SDS)
Thermomixer
Qiagen RNeasy Plus Mini Kit
Vortex Lab Mixer
Centrifuge
1.5 mL microcentrifuge tube
Pipettes
Procedure:

1. Centrifuge the immunoprecipitated samples at 10,000 rpm for 30 s at 4 ˚C and discard the supernatant.
2. Resuspend the pelleted beads in 750 μL of 1X RIPA Buffer containing Phosphatase/Protease Inhibitor Cocktail, mix well by pipetting, and centrifuge at 10,000 rpm for 30 s at 4 ˚C. Discard the supernatant.
3. Repeat the washes with RIPA buffer three more times (total of four washes).
4. Elute the RNA-Ago2 protein complexes from beads by adding 100 μL of elution buffer to the beads and vortexing well.
5. Reverse the crosslinking by incubating the eluted complexes at 70 ˚C for 45 min in a thermomixer at 300 rpm.
6. Centrifuge the suspension at 10,000 rpm for 30 s at room temperature and collect the supernatant containing the RNA.
7. Isolate RNA from the supernatant using the Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol.
8. Elute RNA in 50 μL of elution buffer provided with the kit.

Table 2
List of Primers.

Target Gene	Sequence	
UCA1 1.4 kb RT-PCR Primers	Forward:	5′-TGACATTCTTCTGGACAATGAGTCCCATCATC3'
	Reverse:	5′-ATCAGGCATATTAGCTTTAATGTAGGTGGCGATG-3′
UCA1 qRT-PCR Primers	Forward:	5′-TAAAGCCATGCCCATCAGACAGC-3′
	Reverse:	5′-GGGATGGCCATTTGGAAGGAGTG-3′

Vendor: IDT Inc., IA, USA.

7. Reverse Transcription of RNA to cDNA

- **Materials Needed**:

Bio-Rad iScript cDNA synthesis kit
Qiagen miScript II kit
Vortex Lab Mixer
Centrifuge
Bio-Rad T100 Thermal Cycler mL microcentrifuge tube
200 µL microcentrifuge tube
Pipettes
Procedure:

1. Convert isolated RNA to cDNA using a reverse transcription (RT) kit.
2. For UCA1 lncRNA analysis, convert 15 µL of RNA samples to cDNA in a single RT reaction using the Bio-Rad iScript cDNA synthesis kit according to the manufacturer's protocol in a reaction volume of 20 µL in a Bio-Rad T100 Thermal Cycler. The RT conditions are as follows: Initial priming at 25 ˚C for 5 min, RT at 46 ˚C for 20 min, RT- inactivation at 95 ˚C for 1 min, and final hold at 4 ˚C.
3. For let-7 miRNA analysis, convert 12 µL of RNA samples to cDNA in a single RT reaction using the Qiagen miScript II kit according to the manufacturer's protocol in a reaction volume of 20 µL in a Bio-Rad T100 Thermal Cycler. The RT conditions are as follows: Initial incubation for RT at 37 ˚C for 60 min, RT-inactivation at 95 ˚C for 5 min, and final hold at 4 ˚C.

8. Real-Time PCR (RT-PCR) for lncRNA Analysis

- **Materials Needed**:

UCA1 1.4 kb primers (Table 2)
GoTaq Green PCR Master Mix
Nuclease free water
Bio-Rad Gel Doc EZ Gel Documentation System
Vortex Lab Mixer
Centrifuge
Pipettes
Bio-Rad T100 Thermal Cycler
1.5 mL microcentrifuge tube
200 µL microcentrifuge tube
Agarose
Agarose gel tray, 7 × 7 cm
1.5 mm thick 8-well comb
1X Tris-Acetate-EDTA (TAE) Buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.3)
Measuring Cylinder
250 ml Conical Flask
Microwave Oven
Ethidium Bromide
Bio-Rad Mini-Sub Cell GT Horizontal Electrophoresis System
Procedure:

1. With 1400 nucleotides in length, the presence of UCA1 in Ago2 immunecomplex can be visualized by agarose gel electrophoresis. Use the cDNA synthesized in the previous step for RT-PCR for UCA1.
2. RT-PCR was carried with GoTaq Green PCR Master Mix in a total volume of 25 µl using 5 µl of cDNA per reaction, according to manufacturer's protocol using the eluates from the IgG-, and Ago2-immunoprecipitated samples as well as the Cell Lysate Input in a Bio-Rad T100 Thermal Cycler. The PCR conditions are as follows: Initial denaturation at 94 ˚C for 3 min, followed by 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 54 ˚C for 30 s, elongation at 72 ˚C for 1 min, and final elongation at 72 ˚C for 10 min.

Table 3
List of Let-7 Primers.

miRNA	miRNA Sequence (Obtained from miRBase)	Gene Globe ID (Qiagen)	Comments
hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU	MS00031220	
hsa-let-7a-2–3p	CUGUACAGCCUCCUAGCUUUCC	MS00037205	The proprietary let-7- forward primers and universal reverse primer are from Qiagen, MD, USA
hsa-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU	MS00003122	
hsa-let-7b-3p	CUAUACAACCUACUGCCUUCCC	MS00008281	
hsa-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU	MS00003129	
hsa-let-7c-3p	CUGUACAACCUUCUAGCUUUCC	MS00045815	
hsa-let-7d-5p	AGAGGUAGUAGGUUGCAUAGUU	MS00003136	
hsa-let-7e-5p	UGAGGUAGGAGGUUGUAUAGUU	MS00031227	
hsa-let-7e-3p	CUAUACGGCCUCCUAGCUUUCC	MS00008316	
hsa-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU	MS00006489	
hsa-let-7f-1–3p	CUAUACAAUCUAUUGCCUUCCC	MS00008323	
hsa-let-7f-2–3p	CUAUACAGUCUACUGUCUUUCC	MS00008330	
hsa-let-7 g-5p	UGAGGUAGUAGUUUGUACAGUU	MS00008337	
hsa-let-7 g-3p	CUGUACAGGCCACUGCCUUGC	MS00008344	
hsa-let-7i-5p	UGAGGUAGUAGUUUGUGCUGUU	MS00003157	
hsa-let-7i-3p	CUGCGCAAGCUACUGCCUUGCU	MS00008351	

Vendor: Qiagen, MD, USA.

3. Prepare 1 % agarose gel by mixing 1 gm of agarose in 100 ml 1X TAE buffer in a 250 ml conical flask and heating in a microwave oven until the agarose is fully dissolved. Add 10 μl of ethidium bromide solution (10mg/ml) to the agarose solution, mix well, pour into the agarose gel casting tray, and place the agarose gel comb for creating the loading wells.
4. Once the agarose gel was set, transfer it into the electrophoresis unit filled with 1X TAE buffer, remove the agarose gel comb, and load the PCR products (25 μl), along with 5 μl each of 100 bp and 1 kb molecular markers (DNA ladders) in the appropriate lanes. Run the gel at 80 V for 30 min.
5. Visualize the gel on a Bio-Rad Gel Doc EZ Gel Documentation System.

9. Quantitative Real-Time PCR (qRT-PCR) for the candidate lncRNA and miRNAs

- **Materials Needed**:

UCA1 qRT-PCR primers (Table 2)
Let-7 miRNA primers (Table 3)
SsoAdvanced Universal SYBR Green Supermix miScript SYBR Green PCR kit
Nuclease free water
Vortex Lab Mixer
Centrifuge
Bio-Rad CFX96 Real Time Thermal Cycler
1.5 mL microcentrifuge tube
Pipettes
Procedure:

1. Use the cDNA synthesized in the previous step for qRT-PCR for UCA1 and let-7 miRNAs.
2. For UCA1 analysis in samples, run qRT-PCR with UCA1 primers and 2 μL of cDNA per reaction using SsoAdvanced Universal SYBR Green Supermix according to the manufacturer's protocol. Quantify the levels of bound UCA1 by calculating the fold change with the difference in Cq value compared to the IgG control sample. The qRT-PCR conditions are as follows: Initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s (two-step qRT-PCR), and final continuous melting curve from 65 °C to 95 °C.
3. For let-7 miRNA analysis, run qRT-PCR with Qiagen let-7 miScript primer assays and 2.5 μL of cDNA per reaction using miScript SYBR Green PCR kit according to the manufacturer's protocol. Quantify the levels of bound let-7 miRNAs by calculating the fold change with the difference in Cq value compared to the IgG control sample. The qRT-PCR conditions are as follows: followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s.

Additional Tips:

- **Optimization of Cross-linking:** Inspect the formaldehyde solution for any change in color or precipitation prior to use for effective crosslinking. The efficiency of formaldehyde crosslinking can vary based on cell type and experimental conditions. Vary the formaldehyde concentration and incubation times to prevent over- or under-crosslinking. Verify that the pH of 2.5 M Glycine is at pH of 7.0 and check for the absence of precipitates at the time of use. Optimal crosslinking conditions can be determined by carrying out a pilot experiment as follows:

Perform the M-Clip assay with varying concentrations of formaldehyde and incubation times.

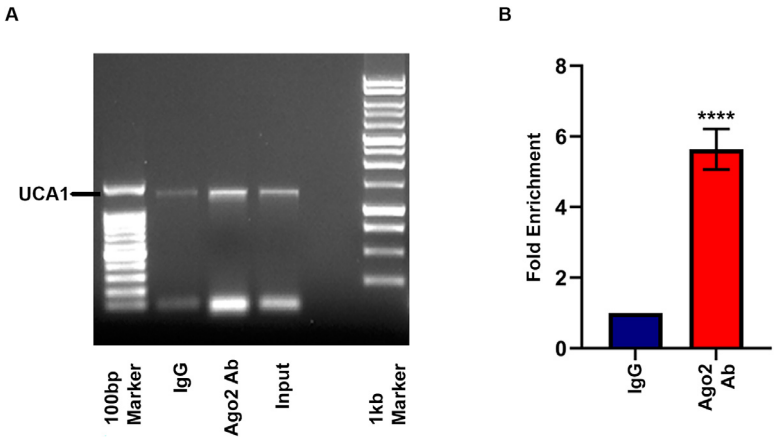

Fig. 2. RT-PCR analysis of Ago2 bound UCA1. A) CLIP assay was performed and RT-PCR was carried out to detect Ago-2-bound UCA1 (1.4 kb) over IgG control, along with UCA1 present in the cell lysate used for M-CLIP assay (input). The RT-PCR products were run on 1 % agarose gels and imaged on a BioRAD Gel-Doc-Ez system. B) The fold enrichments of UCA1 bound to Ago2 was calculated over the IgG controls, are presented graphically. Error bars indicate mean ± SEM. Each of the presented experiments was repeated thrice and the statistical significance between treatment and control groups was determined by Student's *t*-test ($^{**}p < 0.005$; $^{***}p < 0.0005$; $^{****}p < 0.0001$). The agarose gel image is representative of the three sets of experiments performed.

Immunoprecipitate the cross-linked samples with target antibody, isolate the RNA, and conduct qRT-PCR for the target RNA.

Compare RNA yields across the conditions to identify the half-maximal crosslinking point within the linear response range, indicating effective crosslinking with minimal risk of over crosslinking.

- **Optimization of Cell Lysis:** As the lysis conditions may vary with cell type, optimize the RIPA lysis as well as sonication parameters. Insufficient incubation with RIPA buffer as well as sonication may lead to incomplete lysis, while excessive sonication can degrade RNA. Use protease/phosphatase inhibitors during lysis to prevent protein degradation. Add protease and phosphatase to the RIPA lysis buffer just before use to prevent protein degradation.
- **Antibody Selection and Validation:** Use validated antibodies for immunoprecipitation. Poor-quality antibodies can result in weak or non-specific binding. Perform a pilot immunoprecipitation with the antibody to determine optimal concentrations.
- **Washing Steps:** Perform thorough washing of the immunoprecipitated beads to reduce non-specific binding. Avoid vigorous vortexing during washes to prevent loss of beads and bound complexes.
- **Cross-link Reversal:** Carefully control the temperature and duration of the crosslink reversal step to avoid RNA degradation. Verify the efficiency of crosslink reversal by running a small sample on a gel before proceeding with RNA isolation.
- **RNA Isolation and Purity:** Use RNA isolation kits according to the manufacturer's protocol to ensure high RNA yield and purity. Assess RNA integrity using a Bioanalyzer or similar instrument to ensure high-quality RNA for downstream applications. As an added precaution, this assay procedure includes an additional amount of phosphatase inhibitor alongside the standard protease/phosphatase inhibitor cocktail in the lysis buffer. This extra step ensures complete inhibition of phosphatase activity, particularly in samples with elevated phosphatase levels, which could otherwise compromise RNA-protein integrity during the isolation process.
- **qRT-PCR Optimization:** Design specific primers for lncRNAs, and miRNAs and validate them for efficiency and specificity.

Trouble Shooting:

- **Low Yield of Immunoprecipitated RNA:** Increase the amount of starting material or optimize the antibody concentration and incubation times. Multiple reactions may be run for assessing miRNA samples as there are several miRNAs to be assessed for identifying the targets.
- **Non-Specific Binding:** Non-specific interactions during immunoprecipitation can lead to background noise, complicating data interpretation. Increase the stringency of washing steps or use pre-clearing strategies to reduce background.
- **RNA Degradation:** RNA degradation can occur during the various steps of the assay, potentially impacting the final results. Making sure that all reagents and equipment are RNase-free, work quickly on ice to prevent RNA degradation.
- **Technical Replicates:** Perform technical replicates to ensure consistency and reproducibility of results.
- **Documentation:** Document each step meticulously to facilitate troubleshooting and reproducibility.

Method validation

To validate our M-CLIP assay, we conducted experiments using the HGSOC cell line OVCAR8, focusing on the lncRNA, UCA1. We observed significant upregulation of UCA1 in a panel of HGSOC cell lines and patient samples, correlating with its role in promoting

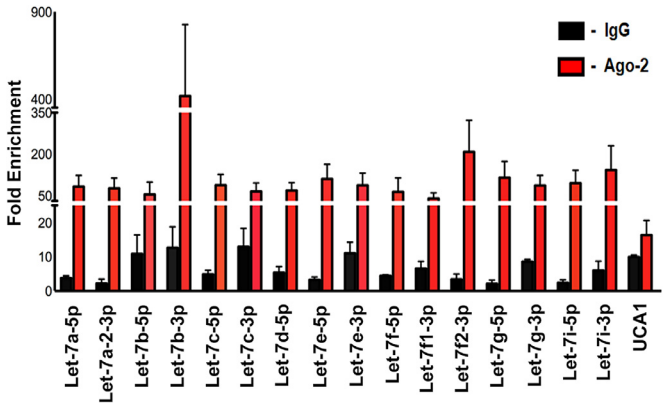

Fig. 3. qRT-PCR analysis of Ago2 bound UCA1 and let-7 miRNAs. CLIP assay was performed and qRT-PCR was carried out to detect Ago-2-bound UCA1 as well as let-7 miRNAs along with respective IgG control. The fold enrichments of UCA1 bound to Ago2 as well as let-7 miRNAs bound to Ago2 were calculated over the IgG controls, are presented graphically. Error bars indicate mean ± SEM. Each of the presented experiments was repeated thrice.

cell proliferation, invasion, migration, and drug resistance [10]. This observation led us to hypothesize that UCA1 acts as a ceRNA by sequestering let-7 miRNAs, thereby enhancing the expression of downstream mRNAs that contribute to cancer progression.

To test this hypothesis, we performed the M-CLIP assay (Fig. 1), targeting Ago2-bound ribonucleoprotein complexes in OVCAR8 cells. In silico analysis using the BiBiServ Portal [11] identified nine distinct binding sites for let-7 miRNAs on UCA1, which guided our focus on these miRNAs during validation. Our M-CLIP assay successfully demonstrated the binding of UCA1 to Ago2 by RT-PCR (Fig. 2) confirming its presence in the ribonucleoprotein complex. Subsequent qRT-PCR analysis validated the presence of UCA1 as well as let-7 miRNAs in the Ago2 complexes (Fig. 3). These results support our hypothesis that UCA1 regulates HGSOC progression by interacting with and quenching let-7 miRNAs, thus preventing them from binding to their mRNA targets.

The findings confirm that UCA1 functions as a ceRNA, contributing to ovarian cancer progression through its interaction with let-7 miRNAs [10]. This validation underscores the effectiveness of the M-CLIP assay in studying lncRNA-miRNA interactions, providing robust and reproducible results. Figures accompanying this section include the immunoprecipitation of Ago2, RT-PCR results demonstrating UCA1 in the Ago2 immune complex (Fig. 2), and qRT-PCR results demonstrating UCA1 lncRNA and let-7 miRNAs in the Ago2 immune complex (Fig. 3). These comprehensive validations highlight the utility of the M-CLIP assay in probing the molecular mechanisms by which lncRNAs influence cancer progression, paving the way for further research and potential therapeutic developments.

Limitations

- The assay is designed to identify interactions but may not accurately quantify the exact number of miRNA molecules sequestered by lncRNAs or determine precise binding affinities. Supplementary quantitative approaches may be necessary for detailed analysis.
- The assay primarily focuses on interactions involving known proteins (e.g., Ago2). It may not capture interactions involving unknown or novel proteins unless they are specifically targeted, potentially limiting the discovery of new interaction partners.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Revathy Nadhan: Data curation, Writing – original draft. **Rohini Gomathinayagam:** Methodology, Data curation. **Rangasudhagar Radhakrishnan:** Methodology, Data curation. **Ji Hee Ha:** Data curation, Writing – review & editing. **Muralidharan Jayaraman:** Data curation, Writing – review & editing. **Danny N. Dhanasekaran:** Conceptualization, Methodology, Supervision, Resources, Funding acquisition, Writing – review & editing.

Data availability

No data was used for the research described in the article.

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