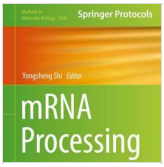

Sep 02, 2021

Robust, Cost-Effective Profiling of RNA Binding Protein Targets with Single-end Enhanced Crosslinking and Immunoprecipitation (seCLIP)

Book Chapter

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ABSTRACT

Profiling of RNA binding protein targets in vivo provides critical insights into the mechanistic roles they play in regulating RNA processing. The enhanced crosslinking and immunoprecipitation (eCLIP) methodology provides a framework for robust, reproducible identification of transcriptome-wide protein-RNA interactions, with dramatically improved efficiency over previous methods. Here we provide a step-by-step description of the eCLIP method, along with insights into optimal performance of critical steps in the protocol. In particular, we describe improvements to the adaptor strategy that enables single-end enhanced CLIP (seCLIP), which removes the requirement for paired-end sequencing of eCLIP libraries. Further, we describe the observation of contaminating RNA present in standard nitrocellulose membrane suppliers, and present options with significantly reduced contamination for sensitive applications. These notes further refine the eCLIP methodology, simplifying robust RNA binding protein studies for all users.

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KEYWORDS

RNA binding protein, CLIP-seq, eCLIP, seCLIP-seq, seCLIP, CLIP, eCLIP, RNA genomics

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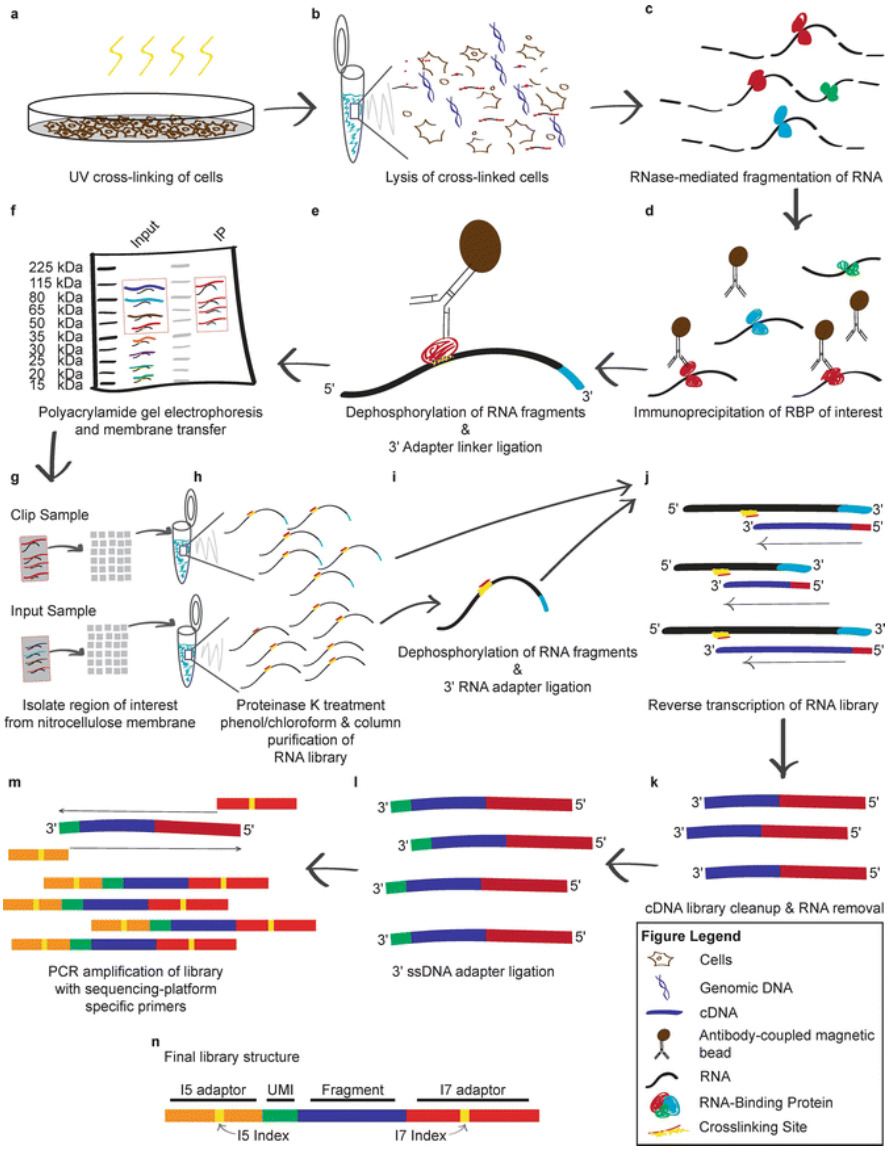

Fig. 1
Schematic of seCLIP method. (**a**) Crosslinking of cultured cells (Subheadings3.1.1and3.1.2). (**b**) Lysis of crosslinked cells (Subheading3.2.1). (**c**) RNA fragmentation with RNase (Subheading3.2.2). (**d**) Immunoprecipitation of RBP -RNA complexes (Subheadings3.3.1–3.3.5). (**e**) Dephosphorylation of RNA fragments and ligation of 3' RNA adapter (Subheading3.5.1). (**f**) Polyacrylamide gel electrophoresis and membrane transfer (Subheadings3.6.1–3.6.5). (**g**) Mince preparative membrane into ~2 mm squares (Subheadings3.6.6) (**h**) RNA isolation from membrane (Subheadings3.6.7and3.6.8). (**i**) Dephosphorylation of RNA fragments and ligation of 3' RNA adapter for input samples (Subheadings3.7.1–3.8.3). (**j**) Reverse transcription of RNA (Subheadings3.9.1and3.9.2). (**k**) cDNA cleanup (removal of excess primers and RNA) (Subheading3.9.3). (**l**) Ligation of 3' DNA adapter (on-bead) and cleanup (Subheadings3.10.1–3.10.3). (**m**) PCR amplification of cDNA library and cleanup (Subheadings3.11.2–3.12.2). (**n**) Final Structure of eCLIP library fragment. The unique molecular identifier or random-mer is shown in green and abbreviated as UMI

MATERIALS TEXT

1. Crosslinking of Cultured Cells

1. 1× DPBS.
2. 254 nM UV crosslinker.
3. Cell scraper.
4. Liquid Nitrogen.

2. seCLIP

1. **Lysis buffer**: 50 mM Tris–HCl pH 7.4, 100 mM NaCl, 1% NP-40 (Igepal CA630), 0.1% SDS, 0.5% sodium deoxycholate

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(protect from light), 1:200 Protease Inhibitor Cocktail III (add fresh), in RNase/DNase-free H_2O.

2. Protease Inhibitor Cocktail III.
3. DNase.
4. RNase I.
5. RNase Inhibitor.
6. Dynabeads M-280 sheep anti-rabbit or Protein A/G magnetic beads.
7. **High salt wash buffer**: 50 mM Tris–HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate (protect from light), in RNase/DNase-free H_2O.
8. **Wash buffer**: 20 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 0.2% Tween-20, in RNase/DNase-free H_2O.
9. **1× TAP Buffer**: 10 mM Tris pH 7.5, 5 mM $MgCl_2$,100 mM KCl, 0.02% Triton X-100, in RNase/DNase-free H_2O.
10. Thermosensitive Alkaline Phosphatase (TAP) (1 unit/µL).
11. **5× PNK pH 6.5 buffer**: 350 mM Tris–HCl pH 6.5, 50 mM $MgCl_2$, in RNase/DNase-free H_2O.
12. 0.1 M DTT.
13. T4 PNK.
14. **1× RNA Ligase Buffer**: 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, in RNase/DNase-free H_2O.
15. 10× Ligase Buffer without DTT.
16. 0.1 M ATP.
17. 100% DMSO.
18. 50% PEG 8000.
19. T4 RNA ligase 1 high concentration.
20. 4–12% Bis-Tris Gel.
21. NuPAGE 4× LDS Sample Buffer.
22. NuPAGE MOPS SDS Running Buffer 20×.
23. NuPAGE Transfer Buffer 20×.
24. PVDF membrane.
25. Nitrocellulose membrane:

A	B	C	D
(a) iBlot 2 Transfer Stacks	ThermoFisher	IB23001	lot #2NR26016-01
or			
(b) Amersham Protran Premium	GE	1060008	lot #G9931040

In 102 K562 and HepG2 eCLIP experiments, an average of 84% of reads uniquely or multiply mapped to the human genome, respectively [11]. However, in preliminary experiments in cell types with decreased RNA yield after membrane transfer (motor neurons (MN) and neural progenitor cells (NPCs) derived from human embryonic stem cells), we observed in some cases more than 90% of sequenced reads were not mapped to the human genome. Using SOAP-denovo [13] to de novo assemble the unmapped reads, we assembled multiple contigs that were queried against the NR database and showed >99% identity to *Acinetobacter johnsonii XBB1* (CP010350.1). Re-mapping these eCLIP datasets revealed millions of reads in many datasets mapping throughout CP010350.1, confirming this specific species as a major contamination source (Fig. 3a). We noted that reads mapping to CP010350.1 had proper CLIP adapter structure, indicating that contamination was likely occurring prior to the 3' linker ligation.

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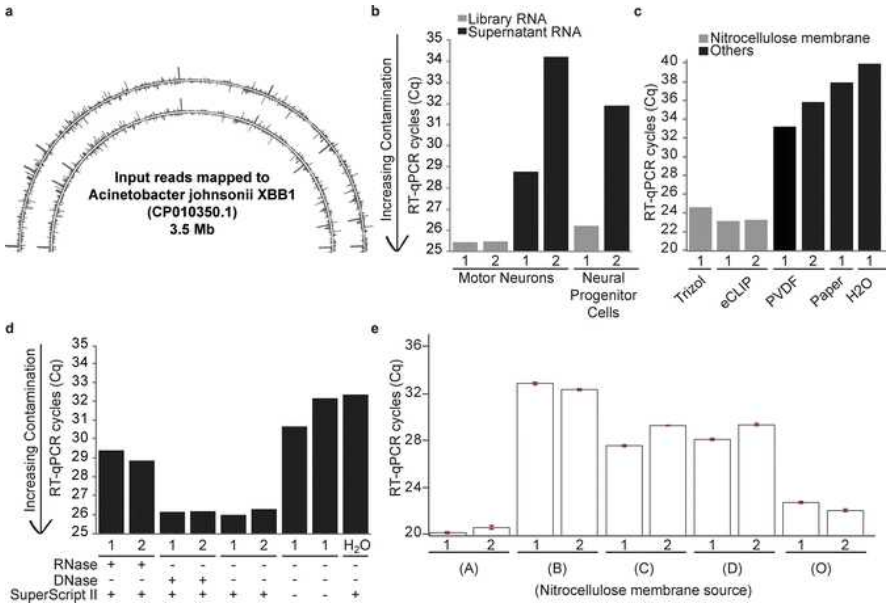

Fig. 3
Genome of Acinetobacter johnsonii XBB1 in nitrocellulose membranes detected by RT—qPCR. (**a**) Sequencing reads from two eCLIP input libraries mapped to Acinetobacter johnsonii XBB1 (CP010350.1). (**b**) Bars indicate Cq from RT—qPCR performed using CP010350.1-specific qPCR primers on eCLIP RNA and supernatant samples from indicated cell types. Lower Cq reflects higher CP010350.1 signal. Numbers indicate replicate experiments. (**c**) Bars indicate RT—qPCR Cq for CP010350.1 from RNA isolated from nitrocellulose membranes via indicated method, PVDF membranes (RNA isolation by Trizol), paper (RNA isolation by Trizol), and H_2O (Trizol extraction). (**d**) Bars indicate RT—qPCR Ct for CP010350.1 from RNA extracted from size-matched nitrocellulose membranes, in technical replicates. Symbols below indicate samples that were either RNase or DNase treated, and those with and without RT enzyme added. (**e**) Bars indicate RT—qPCR Ct for CP010350.1 from RNA extracted from nitrocellulose membrane samples from five sources in technical replicates as follows: (A) commercial source A, (B) ThermoFisher iBlot (IB23001 lot 2NR26016-01), (C) GE Amersham Protran Premium (13600117 lot G6552142), (D) GE Amersham Protran Premium (1060008 lot G9931040), and (O) original commercial source. Error bars indicate standard deviation from RT—qPCR triplicate measurements

In order to modify eCLIP to ameliorate this issue, we set out to identify the source of this contamination by performing RT-qPCR using primers designed against regions of CP010350.1 with high read density. To first confirm that this contamination was not present in initial samples, we extracted RNA (Trizol LS) from supernatant remaining after immunoprecipitation in addition to standard post-membrane transfer and isolation and performed RT-qPCR for bacterial RNA. We observed more than tenfold increased bacterial RNA signal in the membrane-isolated RNA as compared to supernatant RNA from the equivalent number of cells, indicating that the contamination was not present during tissue culture and was introduced during the IP/Western stage (Fig. 3b). Similar RT-qPCR assays performed after RNA isolation on various buffers or enzyme mixes used failed to identify significant contamination (data not shown).

The observation that these reads were only present in input but not CLIP samples (despite input often having more than 100-fold more RNA recovery and library yield [12]), implicated the difference in RNA adaptor ligation of CLIP (3' RNA adaptor ligation on-bead, before the protein electrophoresis step) versus input (3' RNA adaptor ligation after RNA isolation off of membranes) RNA. Surprisingly, we found that RNA extraction (Trizol) of nitrocellulose membrane alone yielded RT-qPCR signal similar to our contaminated libraries, in contrast to the lower signal observed after RNA isolation from PVDF membranes, Whatman and other lab paper, or negative controls (Fig. 3c). We observed identical results with freshly ordered membrane stock (data not shown). To further explore the nature of the contamination, we synthesized cDNA from either RNase or DNase-treated membrane samples and repeated the RT-qPCR assay. This indicated that the contamination was likely RNA, as the RT-qPCR signal was sensitive to both RNase and the no-RT control, but not DNase (Fig. 3d). The strand-specific signal observed in reads similarly implicated RNA contamination (Fig. 3a).

As the nitrocellulose transfer provides key specificity for isolating RNA crosslinked to protein, we set out to identify optimized alternative sources that had decreased RNA background. We obtained four additional nitrocellulose membrane sources (A), (B) ThermoFisher iBlot2 (IB23001 lot 2NR26016-01), (C) GE Amersham Protran Premium (13600117 lot G6552142), and (D) GE Amersham Protran Premium (1060008 lot G9931040), in addition to our original commercial source (O). For each, we performed RNA isolation followed by the bacterial RNA RT-qPCR, and observed that whereas O and A showed similar CP010350.1

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contamination, B, C, and D did not (Fig. 3e). When we prepared libraries according to our standard protocol for eCLIP input samples, we observed that library yields reflected these results, with B, C, and D showing the least amount of overall contamination (data not shown). Importantly, we observed no difference in RNA or library yield when we prepared standard eCLIP input libraries for two protein size ranges of roughly equal membrane size (10–50 kDa and 50–225 kDa) for multiple membrane types. Thus, these results indicate that testing of nitrocellulose membranes enables optimization of eCLIP by removing substantial background contamination. Our results identify (B) ThermoFisher iBlot and (C) GE Amersham Protran Premium (1060008) membranes as options which show a dramatic decrease in contamination for sensitive eCLIP experiments without altering true library yield. Although yielding equally low contamination, (D) GE Amersham Protran Premium (13600117) is "trial-size" packaging and generally commercially unavailable for large-scale use. Other sources can be tested using the RT-qPCR method described here to determine whether they are of sufficiently low background for use in eCLIP.

26. 5% milk + TBST (1× TBS pH 7.4 + 0.05% Tween-20).
27. Rabbit TrueBlot HRP secondary antibody.
28. ECL Western Blotting detection assay.
29. Proteinase K.
30. Urea.
31. Acid Phenol/Chloroform/Isoamylalcohol pH 4.5.
32. Phase lock heavy 2 mL Tubes.
33. 100% Ethanol.
34. RNA Clean & Concentrator-5 Kit.
35. Dynabeads MyOne Silane.
36. RLT Buffer.
37. 5 M NaCl.
38. 10× Ligase Buffer with DTT.
39. 10× AffinityScript reverse transcriptase buffer.
40. AffinityScript reverse transcriptase.
41. dNTPs (25 mM each).
42. Exo-SAP-IT.
43. 0.5 M EDTA.
44. 1 M NaOH.
45. 1 M HCl.
46. 5 mM Tris–HCl pH 7.5.
47. 10 mM Tris–HCl pH 7.5.
48. Q5 or other high fidelity PCR Master Mix.
49. qPCR Master Mix.
50. Agencourt AMPure XP beads.
51. MinElute gel purification Kit.
52. D1000 DNA tape/reagent.

3. Contamination Assay

1. TRIzol®Reagent.
2. TRIzol®LS Reagent.
3. SuperScript II (200 unit/µL).

4. Primer Sequences

seCLIP:

1. InvRiL19: /5Phos/rArGrArUrCrGrGrArArGrArGrCrArCrArCrGrUrC/3SpC3/
(Order 100 nmole RNA oligo, standard desalting; storage stock 200 µM; working stock 40 µM; final concentration 1 µM (input), 4 µM (CLIP)).
2. InvRand3Tr3: /5Phos/NNNNNNNNNNAGATCGGAAGAGCGTCGTGT/3SpC3/
(Order 100 nmole DNA oligo, standard desalting; storage stock 200 µM; working stock 80 µM; final concentration 3 µM).
3. InvAR17: CAGACGTGTGCTCTTCCGA (25 nmole DNA oligo, standard desalting; storage stock 200 µM; working stock 20 µM; final concentration 0.5 µM).
4. D5x_qPCR: AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCT.
5. D7x_qPCR: CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC.

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XBB1 contamination primers:

1. XBB1_qPCR _F: GAGGCGGCAAATATCCTGTG.
2. XBB1_qPCR_R: GTTTCACTTCCCCTCGTTCG.

SAFETY WARNINGS

For health and environmental hazards, please refer to the Safety Data Sheets (SDS).

ABSTRACT

Profiling of RNA binding protein targets in vivo provides critical insights into the mechanistic roles they play in regulating RNA processing. The enhanced crosslinking and immunoprecipitation (eCLIP) methodology provides a framework for robust, reproducible identification of transcriptome-wide protein-RNA interactions, with dramatically improved efficiency over previous methods. Here we provide a step-by-step description of the eCLIP method, along with insights into optimal performance of critical steps in the protocol. In particular, we describe improvements to the adaptor strategy that enables single-end enhanced CLIP (seCLIP), which removes the requirement for paired-end sequencing of eCLIP libraries. Further, we describe the observation of contaminating RNA present in standard nitrocellulose membrane suppliers, and present options with significantly reduced contamination for sensitive applications. These notes further refine the eCLIP methodology, simplifying robust RNA binding protein studies for all users.

FILES

Crosslinking of Cultured Cells
Version 1
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Cell Lysis and RNA Fragmentation
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Capture RBP -RNA Complexes on Beads
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Immunoprecipitation Washes and RNA Dephosphorylation
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Ligate 3' RNA Adapter (On-Bead)
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Western Blotting and RNA Isolation from Membrane
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Dephosphorylation of Input RNA
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3' RNA Adapter Ligation to Input RNA

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Reverse Transcribe RNA (All Clip and Input Samples), and Reaction Cleanup
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3' Linker Ligate cDNA (On-Bead), and Cleanup
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qPCR Quantify cDNA, PCR, and Reaction Cleanup
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Agarose Gel Electrophoresis and Size-Selection of Library
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