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TITLE:

Transcriptome-Wide Profiling of Protein-RNA Interactions by Cross-Linking and Immunoprecipitation Mediated by FLAG-Biotin Tandem Purification

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KEYWORDS:

^{Fbio}CLIP-seq, RNA biology, RNA-binding protein, protein-RNA interaction, WDR43, LIN28

SUMMARY:

Here we present a modified CLIP-seq protocol called ^{Fbio}CLIP-seq with FLAG-biotin tandem purification to determine the RNA targets of RNA-binding proteins (RBPs) in mammalian cells.

ABSTRACT:

RNA and RNA-binding proteins (RBPs) control multiple biological processes. The spatial and temporal arrangement of RNAs and RBPs underlies the delicate regulation of these processes. A strategy called CLIP-seq (cross-linking and immunoprecipitation) has been developed to capture endogenous protein-RNA interactions with UV cross-linking followed by immunoprecipitation. Despite the wide use of conventional CLIP-seq method in RBP study, the CLIP method is limited by the availability of high-quality antibodies, potential contaminants from the copurified RBPs, requirement of isotope manipulation, and potential loss of information during a tedious experimental procedure. Here we describe a modified CLIP-seq method called ^{Fbio}CLIP-seq using the FLAG-biotin tag tandem purification. Through tandem purification and stringent wash conditions, almost all the interacting RNA-binding proteins are removed. Thus, the RNAs interacting indirectly mediated by these copurified RBPs are also reduced. Our ^{Fbio}CLIP-seq method allows efficient detection of direct protein-bound RNAs without SDS-PAGE and membrane transfer procedures in an isotope-free and protein-specific antibody-free manner.

INTRODUCTION:

RNAs and RNA-binding proteins (RBPs) control diverse cellular processes including splicing, translation, ribosome biogenesis, epigenetic regulation, and cell fate transition[1-6]. The delicate

mechanisms of these processes depend on the unique spatial and temporal arrangement of RNAs and RBPs. Therefore, an important step towards understanding RNA regulation at the molecular level is to reveal the positional information about the binding sites of RBPs.

A strategy referred to as cross-linking and immunoprecipitation (CLIP-seq) has been developed to capture protein-RNA interactions with UV cross-linking followed by immunoprecipitation of the protein of interest[7]. The key feature of the methodology is the induction of covalent cross-links between an RNA-binding protein and its directly bound RNA molecules (within ~1 Å) by UV irradiation[8]. The RBP footprints can be determined by CLIP tag clustering and peak calling, which usually have a resolution of 30−60 nt. Alternatively, the reverse transcription step of CLIP can lead to indels (insertions or deletions) or substitutions to the cross-linking sites, which allows identification of protein binding sites on the RNAs at a single-nucleotide resolution. Pipelines like Novoalign and CIMS have been developed for the analysis of the high-throughput sequencing results of CLIP-seq[8]. Several modified CLIP-seq methods have also been proposed, including individual-nucleotide resolution cross-linking and immunoprecipitation (iCLIP), enhanced CLIP (eCLIP), irCLIP, and photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP)[9-12].

Despite the wide use of traditional CLIP-seq methods in the study of RBPs, the CLIP methods have several drawbacks. First, the tedious denatured gel electrophoresis and membrane transfer procedure may lead to loss of information, and cause limited sequence complexity. Second, the protein specific antibody-based CLIP method may pull down a protein complex instead of a single target protein, which may lead to false positive protein-RNA interactions from the copurified RBPs. Third, the antibody-based strategy requires a large amount of high-quality antibodies, which makes the application of these methods inadequate for the study of RBPs without high-quality antibodies available. Fourth, the traditional CLIP method requires radiolabeled ATP to label the protein-bound RNAs.

The high affinity of streptavidin to biotinylated proteins makes it a very powerful approach to purify specific proteins or protein complexes. The efficient biotinylation of proteins carrying an artificial peptide sequence by ectopically expressed bacterial BirA biotin ligase in mammalian cells makes it an efficient strategy to perform biotin purification in vivo[13]. We developed a modified CLIP-seq method called ^{Fbio}CLIP-seq (FLAG-Biotin-mediated Cross-linking and Immunoprecipitation followed by high-throughput sequencing) using FLAG-biotin tag tandem purification[14] (**Figure 1**). Through tandem purification and stringent wash conditions, almost all the interacting RBPs are removed (**Figure 2**). The stringent wash conditions also allow circumventing the SDS-PAGE and membrane transfer, which is labor intensive and technically challenging. And similar to eCLIP and irCLIP, the ^{Fbio}CLIP-seq method is isotope-free. Skipping the gel running and transfer steps avoids the loss of information, keeps authentic protein-RNA interactions intact, and increases the library complexity. Moreover, the high efficiency of the tagging system makes it a good choice for RBPs without high-quality antibodies available.

Here we provide a step-by-step description of the ^{Fbio}CLIP-seq protocol for mammalian cells. Briefly, cells are cross-linked by 254 nm UV, followed by cell lysis and FLAG immunoprecipitation

(FLAG-IP). Next, the protein-RNA complexes are further purified by biotin affinity capture and RNAs are fragmented by partial digestion with MNase. Then, the protein-bound RNA is dephosphorylated and ligated with a 3' linker. A 5' RNA linker is added after the RNA is phosphorylated with PNK and eluted by proteinase K digestion. After reverse transcription, the protein-bound RNA signals are amplified by PCR and purified by agarose gel purification. Two RBPs were chosen to exemplify the ^{Fbio}CLIP-seq result. LIN28 is a well-characterized RNA-binding protein involved in microRNA maturation, protein translation, and cell reprogramming[15-17]. WDR43 is a WD40 domain-containing protein thought to coordinate ribosome biogenesis, eukaryotic transcription, and embryonic stem cell pluripotency control[14,18]. Consistent with previously reported results for LIN28 with CLIP-seq, ^{Fbio}CLIP-seq reveals binding sites of LIN28 on "GGAG" motifs in the microRNA mir-let7g and mRNAs[16,19] (**Figure 3**). WDR43 ^{Fbio}CLIP-seq also identified the binding preference of WDR43 with 5' external transcribed spacers (5'-ETS) of pre-rRNAs[20] (**Figure 4**). These results validate the reliability of the ^{Fbio}CLIP-seq method.

PROTOCOL:

1. Cell line construction

1.1. Clone the gene of interest into a PiggyBac vector pPiggyBac-FLAG-bio-[cDNA of interest]-(Hygromycin-resistant) plasmid that carries a FLAG-biotin epitope[13,21] to express a FLAG-biotin tag fused RBP (^{FB}RBP).

1.2. Cotransfect the ^{FB}RBP expressing vector with the pBase vector into a cell line expressing the BirA enzyme[22].

NOTE: In this study, the ^{FB}RBP gene was transfected into mouse embryonic stem cells (mESCs) carrying an integrated BirA expression vector[21]. Alternatively, the ^{FB}RBP expressing vector can be cotransfected into cells with pBase and pPiggyBac-BirA-V5 together.

1.3. Grow the cells in mESC on gelatinized dishes and maintain in mES culture medium (**Table of Materials**) in 5% CO_2 at 37 °C. Select the transfected cells with hygromycin b (100 µg/mL) for 1 week.

1.4. After drug selection, harvest cells by SDS loading buffer (**Table 1**) and use a Western blot[23] to confirm the tagging and expression of the gene with a FLAG antibody and streptavidin-HRP, respectively.

2. Cross-linking

2.1. Plate ~3 x 10^6 mES cells in a 10 cm plate 1 day before the experiment so that the cells will grow to 70–90% confluence when harvested (~5–10 million cells per sample).

2.2. Remove the medium with a vacuum. Treat the cells with 2 mL of 0.25% trypsin-EDTA for 2 min at room temperature (RT) and quench the trypsin by 4 mL of fresh mESC medium. Transfer

the cells to a 15 mL centrifuge tube. Spin down the cells by centrifugation at 300 x *g* for 3 min at 4 °C and remove the supernatant.

2.3. Suspend the cells with 4 mL of cold PBS and plate the cells back to the 10 cm plate for cross-linking. Cross-link the cells by radiation with 254 nm UV light (set the UV cross-linker with a parameter of 400 mJ/cm^2).

NOTE: For cell monolayers, the cells can be cross-linked with UV light directly on the plate before trypsin treatment.

2.4. Transfer the cross-linked cells to a 15 mL tube. Spin down by centrifugation at 300 x *g* for 3 min at 4 °C and remove the supernatant.

NOTE: The cross-linked cell pellet can be stored at -80 °C until use.

3. Cell lysate preparation

3.1. Lyse the cells with 500 µL of wash buffer A (**Table 1**) freshly supplemented with 1 mM DTT, 1 mM PMSF, 1/500 protease inhibitor cocktail, and 400 U/mL RNase inhibitor. Transfer the cells to an RNase-free 1.5 mL tube. Incubate the cells for 30−60 min on a rotor with gentle rotation at 4 °C.

3.2. Treat the lysate with 30 µL of DNase I at 37 °C for 10 min. Stop the reaction by adding 4 µL of 0.5 M EDTA. Spin down the insoluble pellet by 12,000 x *g* for 20 min at 4 °C and transfer the supernatant to a new prechilled 1.5 mL tube.

NOTE: For immediate use, keep on ice. If the supernatant will not be used immediately, store it at -80 °C until use.

4. FLAG beads preparation

4.1. Add 40 µL of slurry FLAG beads per sample to a fresh 1.5 mL tube.

4.2. Quickly wash the beads with 0.5 mL of ice-cold wash buffer A and spin down by 3,000 x *g* for 2 min at 4 °C.

4.3. Repeat step 4.2 once. Spin down the beads with 3,000 x *g* for 2 min at 4 °C. Carefully remove the buffer with a narrow-end pipette tip. Avoid removing the beads.

5. Immunoprecipitation

5.1. Transfer the cell lysate from step 3.2 to the pre-equilibrated FLAG beads. Rotate all the samples on a roller shaker at 4 °C gently. Incubate the FLAG beads with lysate for 2−4 h or overnight.

5.2. Centrifuge the beads for 2 min at 3,000 x *g* and remove the supernatant.

5.3. Wash the beads with 0.5 mL of prechilled wash buffer A by incubation for 5 min in a rotator at 4 °C, spin down the beads with 3,000 x *g* for 2 min and remove the supernatant. Repeat the wash 1x.

5.4. Repeat the wash 2x as described in step 5.3 with 0.5 mL of prechilled wash buffer B (**Table 1**).

6. Elution with 3x FLAG peptide

6.1. Prepare 3x FLAG elution solution by dissolving 1 mg of 3x FLAG peptide with 5 mL of wash buffer A to a final concentration of 200 ng/mL.

6.2. Spin down the FLAG beads from step 5.4 with 3,000 x *g* for 2 min. Remove the supernatant carefully. Make sure most of the supernatant is removed by using a narrow end pipette tip.

6.3. Add 200 µL of 3x FLAG elution solution to each sample. Incubate the samples with gentle rotation for 30 min at 4 °C. Spin down the FLAG beads for 2 min at 3,000 x *g*. Transfer the supernatants to fresh tubes.

6.4. Repeat the elution 2x as described in steps 6.2 and 6.3 and pool the eluents together. Save 5% of the eluents for Western blot analysis or silver staining to check the efficiency of FLAG-IP.

7. Streptavidin beads preparation

7.1. Prepare 50 µL of a streptavidin bead slurry for each sample. Collect the beads with a magnetic stand for 30 s and remove the supernatant with a pipette tip.

7.2. Quickly wash the beads with 0.5 mL of ice-cold wash buffer A and collect the beads with a magnetic stand for 30 s.

7.3. Repeat the wash 1x. Remove the supernatant after the wash.

8. Biotin affinity purification

8.1. Transfer the pooled eluents from step 6.4 to the streptavidin beads from step 7.3 and gently rotate the samples at 4 °C for 1−3 h or overnight.

8.2. Collect the beads with a magnetic stand for 30 s and remove the supernatant. Wash the beads 2x with 500 µL of wash buffer C (**Table 1**) by gentle rotation at RT for 5 min.

8.3. Collect the beads with the magnetic stand and remove the supernatant. Wash the beads 2x

with 500 µL of wash buffer D (**Table 1**) by rotating at RT for 5 min.

8.4. Collect the beads with the magnetic stand and remove the supernatant. Quickly wash the beads 2x with 500 µL of ice-cold PNK buffer (**Table 1**).

9. Partial RNA digestion

9.1. Make a 10^5-fold dilution of MNase with MNase reaction buffer (**Table 1**). Add 100 µL of MNase solution to each sample. Vortex the beads at 37 °C with a thermal mixer set at 1,200 rpm (5 s run, 30 s stop) for 10 min, collect the beads with a magnetic stand for 30 s and remove the supernatant.

NOTE: The concentration of MNase should be optimized for different RNA-binding proteins. The MNase concentration that can digest RNAs into 30−50 nt fragments (determined by the size of insert of the final library) is optimal.

9.2. Quickly wash the beads 2x with 500 µL of ice-cold PNK+EGTA buffer (**Table 1**). Collect the beads with a magnetic stand and remove the supernatant between each wash step.

9.3. Wash the beads 2x with 500 µL of wash buffer C by gentle rotation for 5 min at RT. Then quickly wash the beads 2x with 500 µL of ice-cold PNK buffer.

10. Dephosphorylation of RNA

10.1. Make a calf intestine phosphatase (CIP) reaction mix with 8 µL of 10x CIP buffer (**Table of Materials**), 3 µL of CIP enzyme, and 69 µL of water. The total volume is 80 µL per reaction.

10.2. Collect the beads with a magnetic stand and remove the buffer. Add the CIP reaction mix to the beads. Vortex the beads at 37 °C with a thermal mixer set at 1,200 rpm (5 s run, 30 s stop) for 10 min.

10.3. Quickly wash the beads 2x with 500 µL of ice-cold PNK+EGTA buffer. Quickly wash the beads 2x with 500 µL of ice-cold PNK buffer.

11. 3’ linker ligation

11.1. Make a 3’ linker mix with 4 µL of 20 µM 3’ linker, 4 µL of 10x T4 RNA ligase buffer, 4 µL of 50% PEG8000, 2 µL of T4 RNA ligase 2 (truncated), and 26 µL of water. The total volume is 40 µL per reaction.

11.2. Collect the beads from step 10.3 with a magnetic stand and remove the buffer. Add 40 µL of the 3’ linker ligation mix to the beads. Vortex the beads at 16 °C with a thermal mixer set at 1,200 rpm (5 s run, 30 s stop) for 3 h or overnight.

11.3. Quickly wash the beads 2x with 500 µL of ice-cold PNK+EGTA buffer. Quickly wash the beads 2x with 500 µL of ice-cold PNK buffer.

12. PNK treatment

12.1. Make a PNK mix with 4 µL of 10x PNK buffer, 2 µL of T4 PNK enzyme, 1 µL of 10 mM ATP, and 33 µL of water. The total volume is 40 µL per reaction.

12.2. Collect the beads with a magnetic stand and remove the buffer. Add 40 µL of PNK mix to the beads. Vortex the beads gently at 37 °C with a thermal mixer set at 1,200 rpm (5 s run, 30 s stop) for 10 min.

12.3. Quickly wash the beads with 500 µL of ice-cold PNK+EGTA buffer.

12.4. Quickly wash the beads with 500 µL of ice-cold PNK buffer. Save 5% of the beads for Western or silver staining analysis to confirm the efficiency of immunoprecipitation.

13. RNA isolation

13.1. Collect the beads from step 12.4 with a magnetic stand and remove the buffer. Add 200 µL of proteinase K digestion buffer (**Table 1**). Vortex the beads for 30 min at 37 °C with a thermal mixer set at 1,200 rpm (5 s run, 30 s stop). Magnetically isolate the beads and take the supernatant for the experiment.

13.2. Add 400 µL of RNA isolation reagent (**Table of Materials**) to the supernatant from step 13.1, mix thoroughly, and incubate on ice for 5 min. Add 80 µL of chloroform and mix thoroughly and then incubate on ice for 5 min. Spin down with 12,000 x *g* for 10 min 4 °C.

NOTE: This step should be performed in a chemical hood.

13.3. Take the supernatant (~500 µL) and add two volumes of isopropanol:ethanol mix (1:1), 1/10 volume of 3 M sodium acetate (pH = 5.5), and 1 µL of glycogen. Freeze at -20 °C for 2 h. Centrifuge at 4 °C with 12,000 x *g* for 20 min.

13.4. Wash the pellet 2x with ice-cold 70% ethanol. Spin down the pellet at 4 °C with 12,000 x *g* for 5 min. Remove the supernatant and dry the pellet. Dissolve the RNAs in 5 µL of RNase-free H_2O.

14. 5' RNA linker ligation

14.1. Make a 5' RNA ligation mix with 1 µL of 10x T4 RNA ligase buffer, 0.5 µL of BSA (1 µg/µL), 1 µL of 10 mM ATP, 1 µL of RNase inhibitor, and 0.5 µL of T4 RNA ligase. The total volume is 4 µL per reaction.

14.2. Add 1 µL of 5’ 20 µM RNA linker to the RNA from step 13.4, denature the RNA by heating at 70 °C for 2 min, and chill on ice immediately.

14.3. Add the 5’ RNA ligation mix to the RNA from step 14.2. Incubate at 16 °C overnight.

15. Reverse transcription

15.1. Purify the RNAs as described in section 13 and dissolve the RNA with 11.5 µL of RNase-free water.

15.2. Add 1 µL of 10 µM reverse transcription primer to the purified RNA, heat at 70 °C for 2 min, and chill on ice immediately.

15.3. Make a reverse transcription mix with 4 µL of 5x reverse transcription buffer, 1 µL of 10 mM dNTPs, 1 µL of 0.1 M DTT, 1 µL of RNase inhibitor, and 0.5 µL of reverse transcriptase (**Table of Materials**). The total volume is 7.5 µL per reaction.

15.4. Add 7.5 µL of reverse transcription mix to the RNA, incubate at 50 °C for 30 min, and stop the reaction by incubation at 70 °C for 10 min. Leave at 4 °C.

16. PCR amplification

16.1. Make a PCR amplification mix with 15 µL of 2x PCR master mix (**Table of Materials**), 5 µL of cDNA from step 15.4, 0.6 µL of 10 µM forward primer 1 (**Table 2**), 0.6 µL of 10 µM reverse primer 1 (**Table 2**), and 8.8 µL of H_2O. The total volume is 30 µL.

16.2. Amplify the cDNA with the following settings: 98 °C 30 s, 98 °C 10 s, 60 °C 30 s, 72 °C 30 s (repeat steps 2−4 for 15−20 cycles), 72 °C 2 min, and hold at 4 °C. Run the PCR product on a 2−3% agarose gel. Cut out the DNA of ~100−150 bp, extract DNA with gel extraction kit (**Table of Materials**), and elute the DNA with 20 µL of water.

16.3. Take 3 µL of the purified PCR product, amplify with forward primer 2 (**Table 2**) and reverse primer 2 (index primer; **Table 2**) as described in step 16.2 for five cycles to introduce index sequences. Purify the PCR product as described in step 16.2.

16.4. Sequence the library with a high-throughput sequencing platform.

17. Bioinformatics analysis

17.1. Perform data analysis using commercial programs as previously described[8].

REPRESENTATIVE RESULTS:

The schematic representation of the ^{Fbio}CLIP-seq procedure is shown in **Figure 1**. Compared with FLAG-mediated or streptavidin-mediated one-step affinity purification, FLAG-biotin tandem

purification removed almost all the copurified proteins, avoiding the contamination of indirect protein-RNA interactions (**Figure 2**). Representative results for ^{Fbio}CLIP-seq for LIN28 and WDR43 are depicted in **Figure 3** and **Figure 4**. We performed LIN28 or WDR43 ^{Fbio}CLIP-seq with mESCs. **Figure 3A** shows the track view of LIN28 and WDR43 ^{Fbio}CLIP-seq in pre-let-7g. The reported GGAG motif in pre-let-7g and the cross-linked sites identified by ^{Fbio}CLIP-seq are shown in **Figure 3B**. The classification of the mutation sites called by CIMS algorithm showed that LIN28 prefers to bind and cross-link to G nucleotide (**Figure 3C**). The enriched RNA motifs in LIN28 ^{Fbio}CLIP-seq binding sites are shown in **Figure 3D**. More representative tracks of ^{Fbio}CLIP-seq on LIN28 and HNRNPU are shown in **Figure 3E**. Comparison of WDR43 and LIN28 ^{Fbio}CLIP-seq showed their different binding patterns in Rn45 pre-rRNA locus (**Figure 4**). **Figure 5** shows the representative results of FLAG and biotin tag validation after cell line construction. **Figure 6** shows representative results of efficient (**Figure 6A**) or unsuccessful (**Figure 6B**) FLAG-IP and FLAG-biotin tandem purification.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of ^{Fbio}CLIP. UV-cross-linked cells (step 1) are lysed in lysis buffer (step 2). The ^{FB}RBP-RNA complex is immunoprecipitated using anti-FLAG resin (steps 3−4). The eluted protein-RNA complex is further purified with streptavidin beads and stringent wash conditions are used to remove protein-protein interactions (steps 5−6). RNAs are partially digested by MNase and non-protein-bound RNA fragments are removed by further wash (steps 7−8). The RNAs are then dephosphorylated and ligated with 3’ linker (step 9). Then the RNAs are phosphorylated and eluted by proteinase K treatment (step 10). Purified RNAs are ligated with a 5’ RNA linker containing a 6 nt random barcode (step 11). After reverse transcription (step 12), the cDNA library is amplified with PCR and high-throughput sequencing is performed (steps 13−15).

Figure 2: FLAG-biotin tandem purification removes copurified proteins. Silver staining of ^{FB}WDR43 showed that almost all the interacting proteins presented in FLAG- or biotin-mediated one-step purification were eliminated after stringent wash in tandem purification. FLAG: FLAG-mediated affinity purification, SA: streptavidin-mediated biotin purification, tandem: FLAG-biotin tandem purification.

Figure 3: Representative results of LIN28 ^{Fbio}CLIP-seq. (**A**) Track view of LIN28 ^{Fbio}CLIP-seq tags and mutation reads called by CIMS in pre-let-7g RNA. The tags shown are unique reads of ^{Fbio}CLIP-seq. In total, ~7.7 million unique reads for LIN28 ^{Fbio}CLIP-seq and ~2.2 million unique reads for WDR43 ^{Fbio}CLIP-seq were retrieved after removing redundant reads. (**B**) Cross-linked sites on the GGAG motif of pre-let7-g RNA by LIN28 ^{Fbio}CLIP-seq. The arrows indicate the cross-linked sites. (**C**) Percentage of mutated nucleotides in different types of mutations. G is the most frequent cross-linked and mutated nucleotide. Random: random distribution of the four nucleotides. (**D**) Predicted LIN28 binding motifs by ^{Fbio}CLIP-seq with HOMER algorithm. (**E**) Track views of LIN28 ^{Fbio}CLIP-seq on LIN28 and HNRNPU mRNAs.

Figure 4: Representative results of WDR43 and LIN28 ^{Fbio}CLIP-seq on Rn45S locus. Track view of WDR43 and LIN28 ^{Fbio}CLIP-seq on Rn45S locus. WDR43 and LIN28 showed different binding

patterns on pre-rRNA.

Figure 5: Representative results of FLAG and biotin tag validation. FLAG or biotin Western blot validates the tagging of the cell lines. Clones #1, #2, and #4 showed efficient expression and biotinylation of the tag while #3 showed poor expression of the tagged protein.

Figure 6: Representative results of FLAG immunoprecipitation and tandem purification efficiency validation. (**A**) Example of efficient purification of tagged protein by FLAG and biotin affinity purification. (**B**) Example of inefficient purification of tagged protein by FLAG and biotin affinity purification.

Table 1: Composition of buffers used in this study.

Table 2: Oligonucleotides used in this study.

DISCUSSION:

Here we introduce a modified CLIP-seq method called ^{Fbio}CLIP-seq, taking advantage of the FLAG-biotin double tagging system to perform tandem purification of protein-RNA complexes. The FLAG-biotin double tagging system has been shown to be powerful in identifying protein-protein and protein-DNA interactions[13,21]. Here we demonstrate the high specificity and convenience of this system in identifying the RNAs interacting with proteins. Through tandem purification and stringent wash conditions, we skipped the challenging SDS-PAGE and membrane transfer step, so that more protein-RNA interactions were preserved and to avoid contaminated RNA signals mediated by RBPs in the same complex. Besides, bypass of these steps avoids labeling of the RNA with radiolabeled ATP. This makes the procedure much easier. Note that during the preparation of our publication, a similar method called uvCLAP has also been proposed[24].

Several steps are important for the success of the protocol. First, the efficiency of the biotinylation of the tagged protein should be confirmed by Western blot before the experiment (**Figure 5**). Second, the efficient elution of purified protein by is required for the production of the RNA signals. The beads from step 12.4 should be analyzed with Western blot or silver staining to guarantee that a decent amount of target protein is retrieved (**Figure 6**). To increase the efficiency, either increase the 3 x FLAG peptide concentration in step 6 up to 500 ng/mL or repeat the elution step multiple times to get better production. Third, it is optimal to titrate the MNase concentration for each protein at the first trial. Overdigestion or insufficient treatment may lead to RNA fragments of inappropriate sizes. Last, the protocol contains two rounds of affinity purification and highly stringent wash. Only a small amount of purified RNAs is retrieved. The reagent should be RNase-free and avoid RNA degradation after MNase treatment step.

Despite the ease of this method, there are still some aspects that can be improved. For example, ligation of a 5’ adaptor to the RNA directly may limit the recovery of the signals because a significant portion of reverse transcription will be terminated by residual cross-linked peptides. Our current study is mainly based on ectopic expression of tagged proteins, which may lead to some artifacts due to protein overexpression. It is worth improving the method by introducing

the tag into the endogenous locus. The CRISPR/Cas9 system makes cell line construction much easier and doable. Some studies have applied the eCLIP-seq experiment to mouse tissues[25]. Derivation of knock-in mice with the double tag is also a potential and promising direction in the improvement and application of the ^{Fbio}CLIP-seq method. Furthermore, ectopically expressed bacterial BirA ligase may lead to unexpected biotinylation events in vivo. Tagging of the protein may also affect its biological functions.

A large number of studies have shown that the gene expression and epigenome of cells are heterogeneous instead of fully homogeneous, suggesting that it is valuable to study the interaction network at a single-cell level. Several strategies have been developed to study the transcriptome and epigenome of single cells. However, no strategies have been reported to analyze the protein-RNA interactome at a single-cell level. Without the denatured gel running and membrane transfer steps, ^{Fbio}CLIP-seq can preserve more signals, which makes it a potential strategy to study protein-RNA interactions at a single-cell level.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

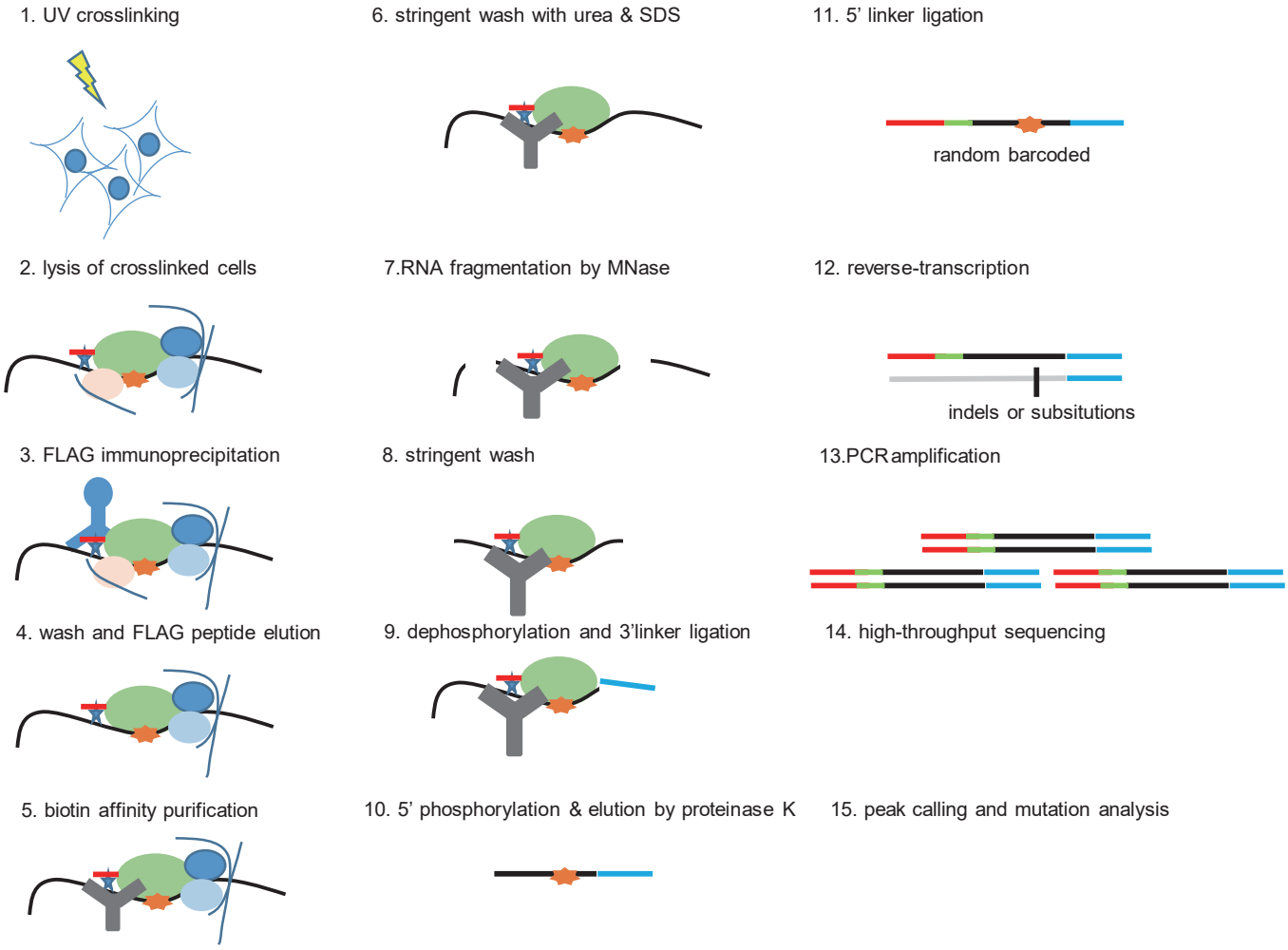
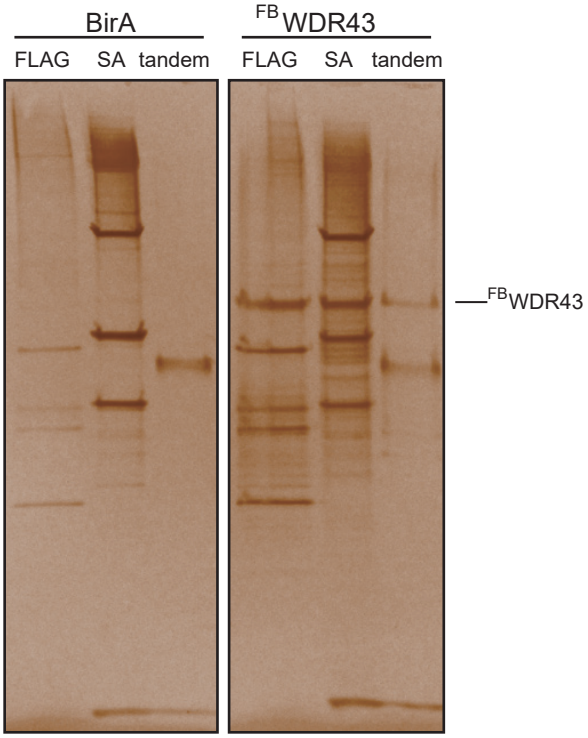


Figure 2



silver staining

Figure 3

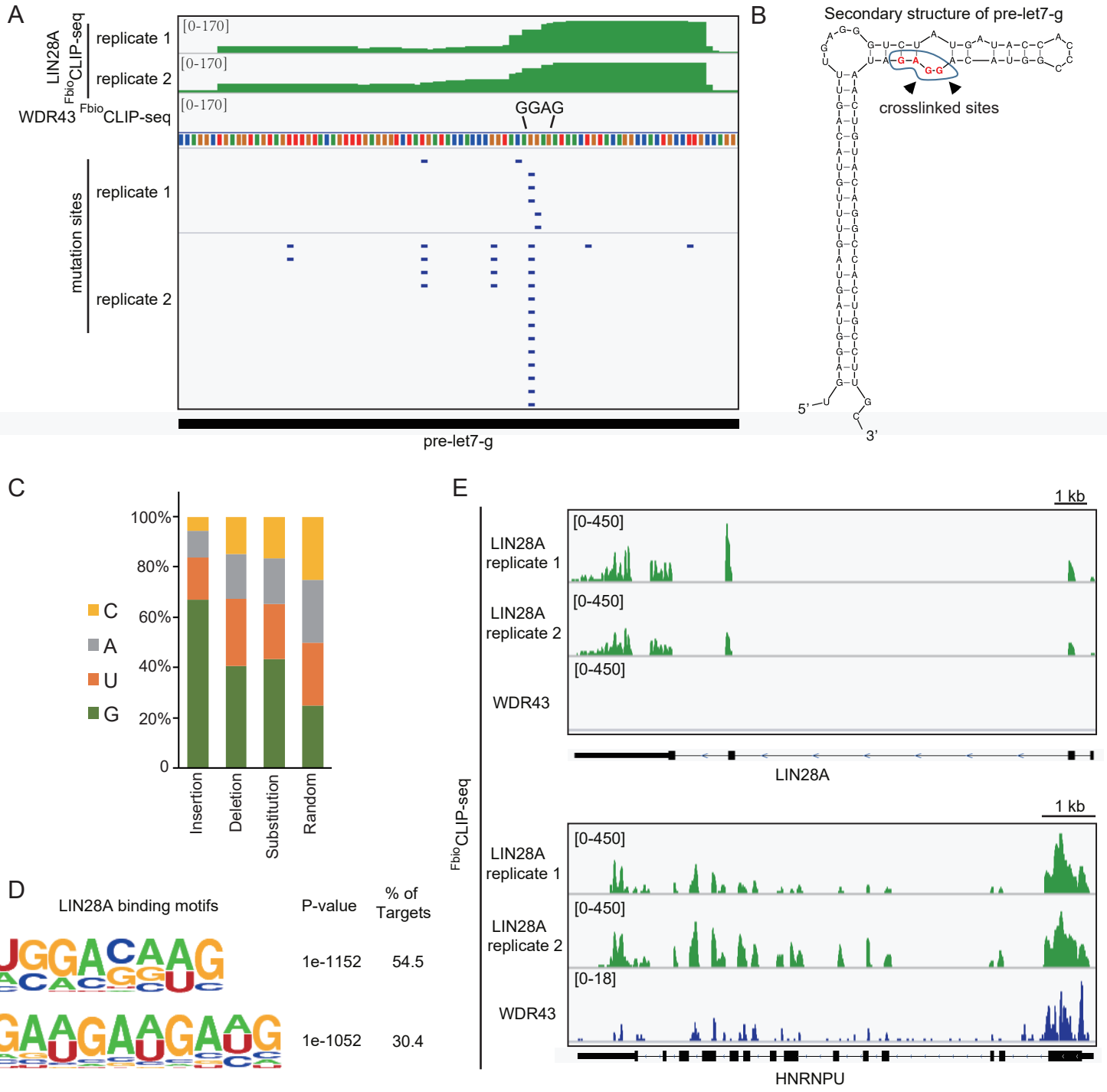
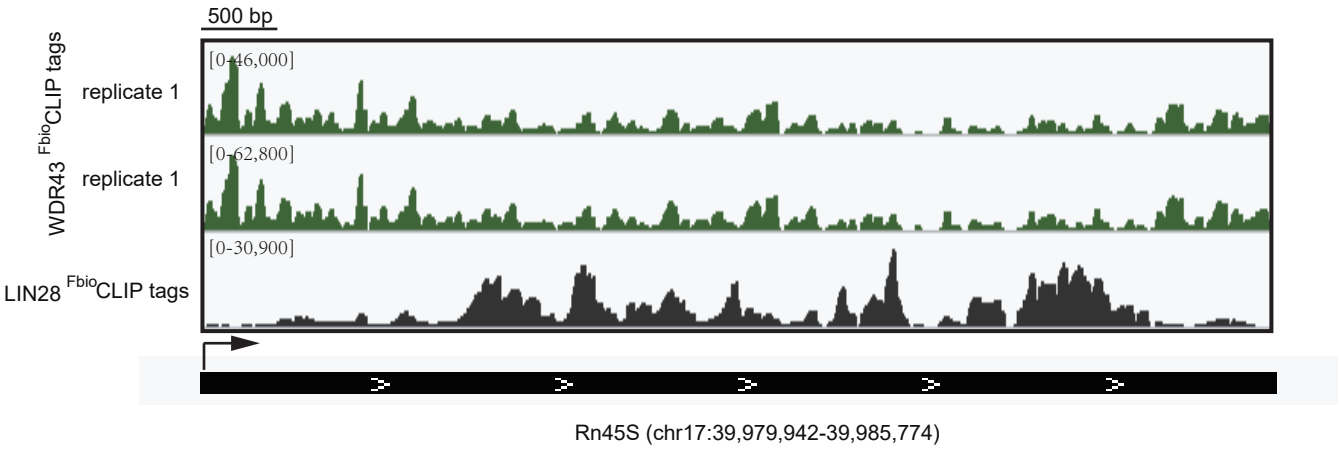


Figure 4



Rn45S (chr17:39,979,942-39,985,774)

Figure 5

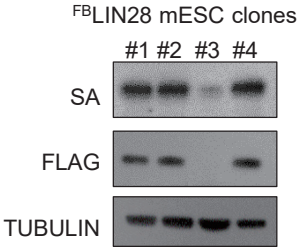


Figure 6

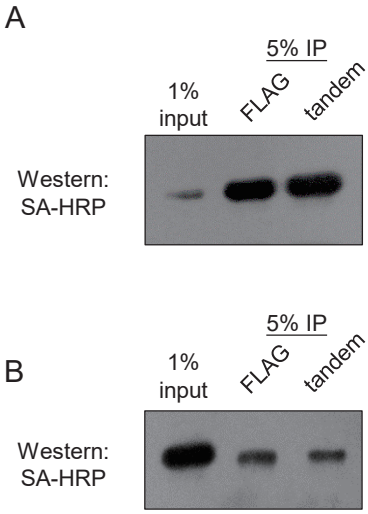


Table 1

Buffer	**Composition**
SDS loading buffer	50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenolblue, 10% glycerol, 100 mM DTT
Wash buffer A	1x PBS, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS
Wash buffer B	5x PBS, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS
Wash buffer C	50 mM Tris pH 7.4, 2% SDS
Wash buffer D	5x PBS, 0.5% NP-40, 0.5% SDS, 1 M urea
PNK buffer	50 mM Tris pH 7.4, 0.5% NP-40, 10 mM $MgCl_2$
MNase reaction buffer	10 mM Tris pH 8.0, 1 mM $CaCl_2$
PNK+EGTA buffer	50 mM Tris pH 7.4, 0.5% NP-40, 10 mM EGTA
Proteinase K digestion buffer	50 mM Tris pH 7.4, 10 mM EDTA, 50 mM NaCl, 0.5% SDS, 20 µg of proteinase K

Table 2

Oligo name	Sequence
3' linker	rAppAGATCGGAAGAGCACACGTCT-NH_2
5' RNA linker	GUUCAGAGUUCUACAGUCCGACGUCNNNNN
RT primer	AGACGTGTGCTCTTCCGATCT
Forward primer 1	GTTCAGAGTTCTACAGTCCGACGATC
Reverse primer 1	AGACGTGTGCTCTTCCGATCT
Forward primer 2	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGAC
Reverse primer 2	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T

Notes

The red and underlined sequences represent Illumina index sequence.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Equipment			
UV crosslinker	UVP	HL-2000 HybrilLinker	
Affinity Purification Beads			
ANTI-FLAG beads	Sigma-Aldrich	A2220	
Streptavidin beads	Invitrogen	112.06D	
Reagents			
10x PBS	Gibco	70013032	
3 M NaOAc	Ambion	AM9740	
3 x FLAG peptide	Sigma-Aldrich	F4799	
ATP	Sigma-Aldrich	A6559	
Calcium chloride ($CaCl_2$)	Sigma-Aldrich	C1016	
CIP	NEB	M0290S	CIP buffer is in the same package.
DTT	Sigma-Aldrich	D0632	
EDTA	Sigma-Aldrich	E9884	
EGTA	Sigma-Aldrich	E3889	
Gel purification kit	QIAGEN	28704	
Glycogen	Ambion	AM9510	
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	449172	
MNase	NEB	M0247S	
NP-40	Amresco	M158-500ML	
PMSF	Sigma-Aldrich	10837091001	
Porteinase K	TAKARA	9033	
Protease inhibitor cocktail	Sigma-Aldrich	P8340	
Q5 High-Fidelity 2X Master Mix	NEB	0492S	

reverse trancriptase (SupperScriptIII)	Invitrogen	18080093	
RNA isolation reagent (Trizol)	Invitrogen	15596018	
RNase Inhibitor	ThermoFisher	EO0381	
RNaseOUT	Invitrogen	10777019	
RQ1 Dnase	Promega	M6101	
SDS	Sigma-Aldrich	1614363	
Sodium chloride	Sigma-Aldrich	S9888	
Sodium deoxycholate	Sigma-Aldrich	D6750	
T4 PNK	NEB	M0201S	PNK buffer is in the same package.
T4 RNA ligaes	ThermoFisher	EL0021	T4 RNA ligase buffer and BSA are in the same package.
T4 RNA ligase2, truncated	NEB	M0242S	T4 RNA ligase buffer and 50% PEG are in the same package.
Trypsin-EDTA	ThermoFisher	25200072	
Urea	Sigma-Aldrich	208884	
mESC culture medium			
DMEM (80%)	Gibco	11965126	
2-Mercaptoethanol	Gibco	21985023	
FCS (15%)	Hyclone		
Glutamax (1%)	Gibco	35050061	
LIF			purified recombinant protein; 10,000 fold dilution
NEAA (1%)	Gibco	11140050	
Nucleoside mix (1%)	Millipore	ES-008-D	
Penicillin-Streptomycin (1%)	Gibco	15140122	
Kit			
DNA gel extraction kit	QIAGEN	28704	

Response to Reviewers' comments

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The revised manuscript by Bi & Shen details the protocol for the FbioCLIP-seq method. The clarification that this should be seen as a method pairing to the Mol Cell paper clarifies many issues, and I appreciate the language corrections addressing other concerns.

Major Concerns:

One major remaining concern is the level of detail in figures -

Figure 5D - what are the p-values (and fraction observation) of these motifs?

Response #1

We have added the p-values and percentage of targets for the motifs in Figure 5D.

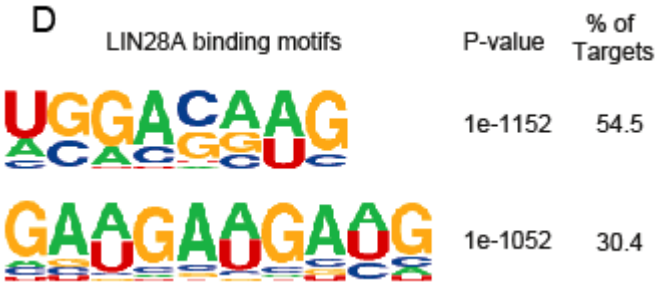
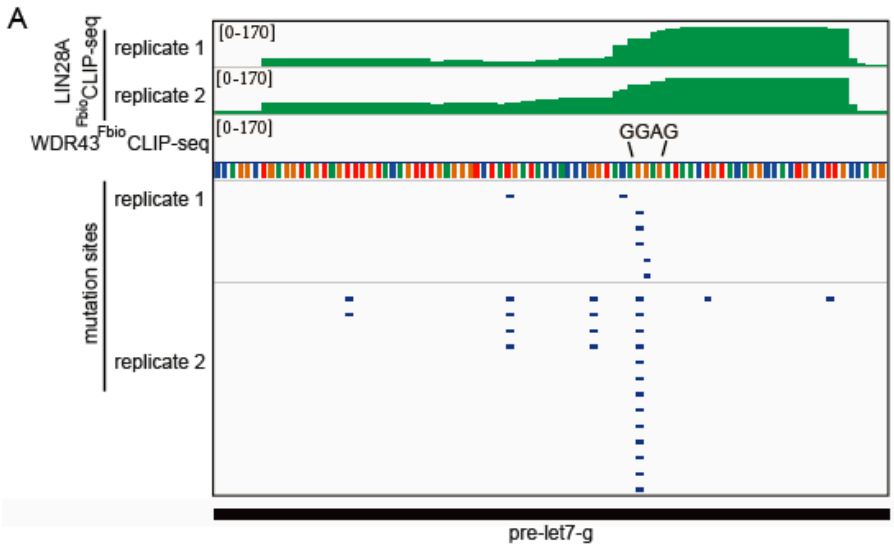


Figure 5A&E - having a non-LIN28 control here would help (I appreciate the addition of LIN28 to figure 6 in response to the prior review) - can the WDR43 data be included here as a negative control? I'm not clear that this figure shows anything without having some non-LIN28A control (whether total RNA-seq, wild-type with no tagged protein, or a different protein).

Response #2

We have added the WDR43 result as a control in the Figures.



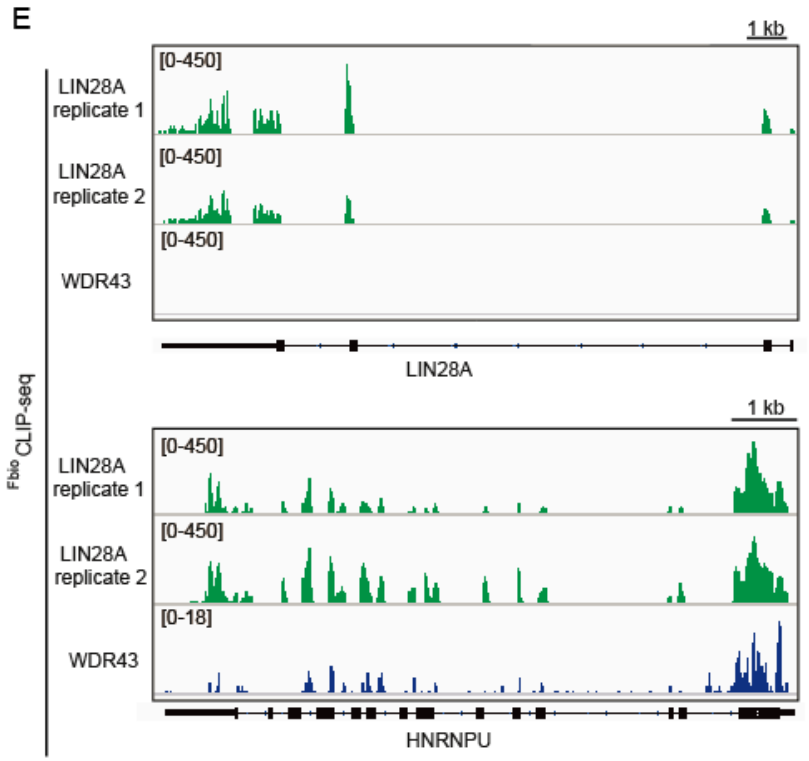


Figure 5A-B - can the 4 positions highlighted in B be added in A? As displayed I'm not clear which bases in B reflect which positions in A.

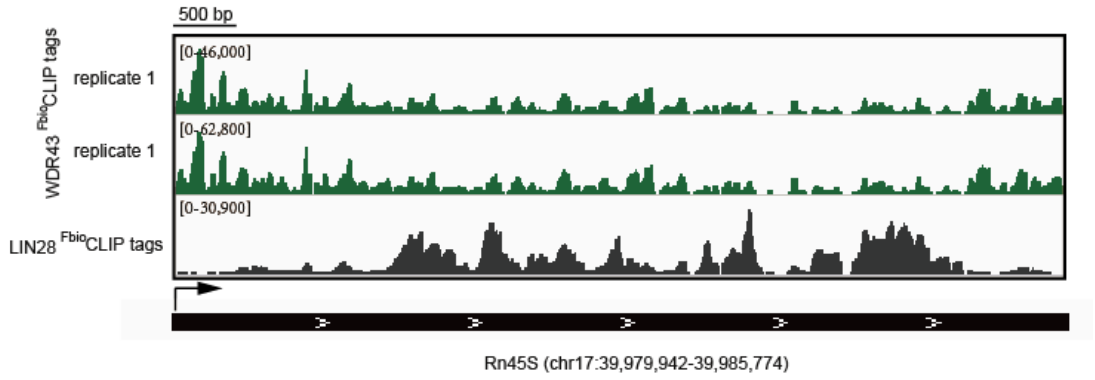
Response #3

The GGAG sequences are labeled in the revised Figure 5A. Please refer to Response #2.

Figure 6 - the positions (x-axis) being shown on the 45S rRNA should be indicated

Response #4

The positions (x-axis) have been indicated by the coordinates of the Rn45S RNA.



Another issue that should be addressed in the text regards the RNA ligations. One of the original insights in iCLIP was that reverse transcription often terminated at the site of crosslinking (and thus these fragments would be lost if the 5' adapter ligation was performed prior to reverse transcription, as was done in original HITS-CLIP methods). As the FbioCLIP method appears to use the HITS-CLIP strategy of 5' RNA linker ligation prior

to reverse transcription, the authors should mention whether they believe this will limit recovery in cases where reverse transcription termination is high (and whether they recommend particular analyses as a result of this design choice), or if their reverse transcription conditions are designed to alleviate this issue.

Response #5:

We appreciate the reviewers comment about the potential limitation of 5' adaptor ligation strategy by HITS-CLIP. We agreed with the comment.

However, we need to point out that despite transcription often terminates at the site of crosslinking, a portion of the reverse transcriptase can overcome the crosslinking "barrier". For these events that successfully cross the "barrier", the reverse transcriptase will stop at the end of RNA molecules instead of the crosslinking site. In such a situation, the iCLIP-seq analysis will treat it as a potential crosslinking site, which actually is a false positive event. For those events fails to overcome the "barrier" of crosslinked peptides, they simply can not be detected by HITS-CLIP or ^{Fbio}CLIP-seq, which is potentially a false negative. In other words, the iCLIP strategy may lead to 'false positive', while HITS-CLIP strategy may lead to 'false negative'. Logically, this limitation of HITS-CLIP strategy can be partially solved by using more input sample to get more RT events that can overcome the "barrier". Still, we agreed the concern of the reviewer and added the statement in the discussion: "Ligation of 5' adaptor to the RNA directly may limit the recovery of the signals because a significant portion of reverse transcription will be terminated by residual crosslinked peptides" (Line 440-442).

Minor Concerns:

I appreciate the response 1.5 with respect to PCR cycles and unique RNA molecules. These numbers (particularly the number of unique reads observed for LIN28A FbioCLIP) would be useful to include in the text to give context for what users of the method should expect if they follow or attempt to repeat the LIN28A experiment as described.

Response #6:

We have added the information in the figure legend.

Reviewer #2:

Manuscript Summary:

Revised manuscript.

Technical concerns were addressed.

Major Concerns:

Missing from Introduction: The strategy behind UV crossing linking, generating mutations, and criteria for a hit identification is missing and needs to be added.

Response #7:

We have added this statement in the text: "The key feature of the methodology is the induction of covalent crosslinks between RNA-binding protein and its directly bound RNA molecules (within ~ 1 Å) by UV irradiation[7]. The RBP footprints can be determined by CLIP tag clustering and peak

calling, which usually have a resolution of 30-60 nt. Alternatively, the reverse transcription step of CLIP may lead to indels (insertions or deletions) or substitutions to the crosslinking sites, which allows a single-nucleotide resolution of the protein binding sites on the RNAs. Pipelines like novoalign and CIMS have been developed for the analysis of the high-throughput sequencing results of CLIP-seq." (Line 43-49). Since the bioinformatics analysis is not the main focus of this protocol, we referred to the available pipelines and the literature for the readers.

Improve names of buffers, at present they are hard to follow
Line 157 'high salt wash buffer"…… 5x PBS or do you mean 1x? is this stringent wash buffer I?
Line 194/5 terminology 'stringent wash buffer I' …stringent wash buffer II'
Make terminology consistent. Ie label each wash buffer I II III or A B C

Response #8:

We have renamed the buffers as suggested by the reviewer to make it easier for reading. And 5x PBS indicates a 5 fold concentration of PBS (~ 750 mM salt).

Need to clean up typos, lack of prepositions.
in-efficient is one word
typo Figure 1 Highthrouput ----------high-throughput

Response #9:

We have revised the typos as suggested.