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Permalink
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Publication Date
2014

DOI
10.1007/978-1-62703-980-2-27

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Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2015 April 28.

Published in final edited form as:

Methods Mol Biol. 2014 ; 1126: 399–410. doi:10.1007/978-1-62703-980-2_27.

Global Protein–RNA Interaction Mapping at Single Nucleotide Resolution by iCLIP-Seq

Chengguo Yao, **Lingjie Weng**, and **Yongsheng Shi**

Abstract

Eukaryotic genomes encode a large number of RNA-binding proteins, which play critical roles in many aspects of gene regulation. To functionally characterize these proteins, a key step is to map their interactions with target RNAs. UV crosslinking and immunoprecipitation coupled with high-throughput sequencing has become the standard method for this purpose. Here we describe the detailed procedure that we have used to characterize the protein–RNA interactions of the mRNA 3′ processing factors.

Keywords

CLIP; iCLIP; UV crosslinking; RNA-binding proteins; High-throughput sequencing

1 Introduction

The human genome encodes more than 800 potential RNA-binding proteins [1], which play a wide variety of important roles in gene expression, including RNA processing, trafficking, translation, and degradation [2]. Functional characterization of these proteins remains a key task in the post-genomic era. A major challenge in this effort has been to identify the natural RNA targets of these proteins in vivo. For this purpose, early studies relied on immunoprecipitation (IP) to isolate specific proteins with their associated RNAs, which are subsequently identified through differential display or microarray analysis (RIP-chip) [3–5]. In some cases, formaldehyde crosslinking was applied prior to IP to capture transient and/or weak protein–RNA interactions [6]. These methods, however, tend to suffer from relatively high background. Additionally, although the RNA targets can be identified by RIP analysis, the specific protein-binding sites within the RNAs could not be mapped [3–6].

To overcome these limitations, the Darnell group pioneered a method called CLIP (UV crosslinking and IP) [7]. In this method, UV irradiation is used to specifically crosslink proteins and RNAs that are in direct contact. Cellular RNAs are then digested into smaller sizes by RNase, and specific proteins and their directly crosslinked RNA fragments are IPed. Following gel purification and linker ligation, the crosslinked RNAs are reverse transcribed and amplified by PCR for sequencing. Compared to RIP analysis, CLIP has a number of important advantages. First, UV irradiation, which only crosslinks proteins and RNAs that are in direct contact, is much more specific than formaldehyde crosslinking. Second, as the

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proteins and their RNA targets are covalently linked, IP can be performed under highly stringent conditions to improve specificity. Third, gel purification enriches the RNAs that are crosslinked to the target proteins instead of other co-purified proteins, further enhancing the specificity. Finally, when coupled with high-throughput sequencing (called HITS-CLIP or CLIP-seq), CLIP allows global mapping of the protein-binding sites at a high resolution. More recently, two modified versions of CLIP, PAR-CLIP (photoactivatable ribonucleoside-enhanced CLIP) and iCLIP (individual-nucleotide resolution CLIP), were introduced that enable global protein–RNA interaction mapping at single nucleotide resolution [8, 9]. We have adopted the iCLIP method developed by the Ule group and successfully applied it to the mRNA 3′ processing factor CstF64 [10]. Here we describe the detailed procedure and offer technical advice on how to optimize it for your protein of interest.

2 Materials

2.1 Solutions

1. 1× PBS buffer: (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4).
2. Cell lysis buffer (50 mM Tris–HCl, pH 7.4; 100 mM NaCl; 1 % NP-40; 0.1% SDS; 0.5 % sodium deoxycholate).
3. High-salt buffer (50 mM Tris–HCl, pH 7.4; 1 M NaCl; 1 mM EDTA; 1 % NP-40; 0.1 % SDS; 0.5 % sodium deoxycholate).
4. PNK buffer (20 mM Tris–HCl, pH 7.4; 10 mM $MgCl_2$; 0.2 % Tween-20).
5. PK buffer (50 mM Tris–HCl, pH 7.4; 50 mM NaCl; 10 mM EDTA).
6. PK-urea buffer (50 mM Tris–HCl, pH 7.4; 50 mM NaCl; 10 mM EDTA; 7 M urea).
7. TE buffer (10 mM Tris–HCl, pH 7.4; 1 mM EDTA).
8. Sodium acetate (3 M, pH 5.2).
9. 100 % Ethanol.
10. NuPAGE MOPS SDS running buffer (Life Technologies).
11. NuPAGE transfer buffer (Life Technologies).
12. RNA phenol/chloroform (MP Biomedicals).
13. 2× SDS gel-loading buffer (100 mM Tris–Cl, pH 6.8; 200 mM β-mercaptoethanol; 2.5 % SDS; 0.2 % bromophenol blue; 25 % glycerol).
14. 2× RNA gel-loading buffer (0.025 % SDS; 0.025 % bromophenol blue; 0.025 % xylene cyanol; 0.5 mM EDTA; 95 % formamide).
15. 8 % Urea-PAGE gel (1× TBE; 8 % polyacrylamide gel [acrylam ide:bisacrylamide = 19:1]; 8 M urea).

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2.2 Enzymes, Reagents, Equipment

1. UV Stratalinker 1800 or equivalent.
2. Protein A Dynabeads (Life Technologies).
3. CstF64 antibody (Bethyl Laboratories).
4. RNase I.
5. DNase I Turbo (Life Technologies).
6. Protease inhibitor cocktail.
7. Shrimp alkaline phosphatase.
8. RNasin.
9. T4 RNA ligase.
10. T4 PNK.
11. [γ-P32]ATP.
12. Pre-stained protein marker.
13. 4–12 % NuPAGE Bis-Tris gel (Life Technologies).
14. Novex mini-cell electrophoresis system (Life Technologies).
15. Nitrocellulose membrane.
16. Thermomixer (Eppendorf).
17. Glycogen.
18. Proteinase K.
19. Superscript III reverse transcriptase.
20. RNase A.
21. Circligase II (Epicentre).
22. FastDigest *Bam* HI (Fermentas).
23. Phusion High-Fidelity DNA polymerase.
24. PCR purification kit (Qiagen).

2.3 Primer Sequences and Linkers

1. RNA linker (Thermo Scientific, formerly Dharmacon).

   5′-Phosphate-AGAUCGGAAGAGCGGUUCAG-3′-puromycin

2. Annealing oligo harboring *Bam* HI restriction enzyme site.

   5′-GTTCAGGATCCACGACGCTCTTCAAAA

3. Reverse transcription primers with different barcodes.

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4. Rclip1: 5′-phosphate-NNAACCNNNAGATCGGAAGAGCGTCGTGGATCCTGAACCGC

   Rclip2: 5′-phosphate-NNACAANNNAGATCGGAAGAGCGTCGTGGATCCTGAACCGC

   Rclip3: 5′-phosphate-NNATTGNNNAGATCGGAAGAGCGTCGTGGATCCTGAACCGC

5. PCR primers

   P5: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

   P3: 5′-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

3 Methods

3.1 UV Crosslinking of Cells/Tissues

For adherent cells

1. Grow cells in 100-mm dishes to 80–90 % confluence.
2. Rinse plates with 1× PBS three times and remove PBS after final wash.
3. Remove lid and place the plate on ice. Irradiate once with 400 mJ/cm^2 at 254 nm in a Stratalinker (or other UV crosslinkers).
4. Add 4 ml 1× PBS to the plate and harvest cells by scraping with a cell lifter. Transfer cell suspension to three 1.5 ml microtubes.
5. Spin down at 15,000 × *g* for 1 min at 4 °C in a minifuge to pellet cells and remove supernatant.
6. Snap-freeze cell pellets on dry ice and store at −80 °C.

For suspension cells

1. Spin down cells at 600 × *g* for 5 min, wash with 1× PBS three times, leave cells in 1× PBS, and transfer to the 100-mm dishes.
2. Place the dishes on ice, remove the lid, irradiate, harvest, and freeze cells as described above.

For tissues

1. Harvest tissue and rinse with cold 1× PBS.
2. Dissociate the tissue by passing through the 200 μl pipette tip.
3. Transfer tissues to 100-mm dishes, irradiate, harvest, and freeze cells as described above.

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3.2 Beads and Cell Lysate Preparation

Beads preparation

1. Use 100 µl of protein A Dynabeads beads (slurry volume) per IP and wash beads twice with 600 µl cell lysis buffer.
2. Resuspend beads in 200 µl cell lysis buffer and add 4–10 µg antibody.
3. Rotate tubes at room temperature for 1 h.
4. Wash beads with 600 µl cell lysis buffer three times.

Cell lysate preparation

1. Resuspend cell pellet in 500 µl cell lysis buffer.
2. Add 5 µl DNase I (2 U/µl), 5 µl protease inhibitor cocktail (100×), and appropriate amount of RNase I (to be determined in pilot experiments).
3. Incubate for 3 min at 37 °C with shaking at 1,200 rpm in a Thermomixer.
4. Transfer to ice and leave on ice for 2 min.
5. Spin down at 15,000 × *g* in 4 °C for 15 min and collect the supernatant for IP.

3.3 Immunoprecipitation

1. Remove wash buffer from beads and mix beads with cell lysate.
2. Rotate the samples overnight at 4 °C.
3. Collect the beads using a magnet and discard the supernatant.
4. Wash beads twice with 600 µl high-salt buffer.
5. Wash beads twice with 600 µl wash buffer.

3.4 Dephosphorylation of the 5′ Ends of RNAs

1. Resuspend beads in

   35 µl water

   4 µl 10× Shrimp alkaline phosphatase buffer

   1 µl Shrimp alkaline phosphatase (10 U/µl)

   Total volume of resuspension reaction: 40 µl.
2. Incubate at 37 °C for 10 min (1,200 rpm for 10 s every half a min in a Thermomixer).
3. Wash beads twice with 600 µl high-salt buffer.
4. Wash beads once with 600 µl PNK buffer.
5. Wash beads once with 50 µl 1× T4 RNA ligase buffer.

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3.5 3′ Linker Ligation

1. Resuspend beads in

   4 μl PEG8000 (50 %)

   4 μl RNA linker (20 μM)

   2 μl 10× T4 RNA ligase buffer

   2 μl BSA (10 μg/μl)

   7 μl water

   0.5 μl RNaseOUT (40 U/μl)

   0.5 μl T4 RNA ligase (10 U/μl)

   Total volume of resuspension reaction: 20 μl.

2. Incubate for 21 h at 16 °C (1,200 rpm for 10 s every 3 min in a Thermomixer).

3. Wash beads with 600 μl PNK buffer twice.

3.6 RNA 5′ End Labeling

1. Resuspend beads in

   15 μl water

   2 μl 10× T4 PNK buffer

   2 μl [γ-P^{32}]ATP (10 μCi/μl)

   1 μl T4 PNK (10 U/μl)

   Total volume of resuspension reaction: 20 μl.

2. Incubate at 37 °C for 10 min (1,200 rpm for 10 s every 3 min in a Thermomixer).

3. Wash beads three times with 600 μl PNK buffer.

3.7 SDS-PAGE and Membrane Transfer

1. Add 20 μl 1× SDS gel-loading buffer to the beads and heat at 70 °C for 5 min.

2. Collect the beads on a magnet and load the supernatant on a NuPAGE gel and load a pre-stained protein marker in the next lane.

3. Run the gel in 1× MOPS running buffer at 180 V until the bromophenol blue dye reaches the bottom of the gel.

4. Transfer the gel to a nitrocellulose membrane using the Novex wet transfer apparatus (400 mA for 1 h at 4 °C).

5. Rinse membrane in 1× PBS, wrap the membrane in plastic wrap and expose it to a phosphorimager screen. To help align the gels with the image, we usually mark the protein ladder bands with a small amount of radioactivity.

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3.8 RNA Isolation and Reverse Transcription

1. Based on the autoradiograph image from the last step (a typical image is shown in Fig. 1, further discussed in Subheading 4), cut out the smear band above the expected protein size (20–70 kDa above the expected protein size). Place the membrane pieces in a 1.5 ml tube.
2. Add 200 μl PK buffer and 10 μl proteinase K (20 μg/μl) to the membrane pieces, incubate at 37 °C for 20 min with shaking at 1,200 rpm in a Thermomixer.
3. Add 200 μl PK-urea buffer, incubate at 37 °C for 20 min with shaking at 1,200 rpm in a Thermomixer.
4. Add 400 μl phenol/chloroform and incubate at 37 °C for 5 min with shaking at 1,200 rpm in a Thermomixer.
5. Spin down at 15,000 × *g* for 10 min.
6. Take the supernatant and add 1 μl glycogen (5 μg/μl), 40 μl 3 M sodium acetate, and 1 ml 100 % ethanol.
7. Incubate overnight in −20 °C.
8. Spin down at 15,000 × *g* for 15 min. Wash pellet with 200 μl 70 % ethanol. Air dry the pellet and dissolve it in 11.5 μl water.
9. Prepare reverse transcription reaction as follows:

   11.5 μl RNA

   1 μl RT primer (2 μM, Rclip1, 2, or 3)

   1 μl dNTPs (10 μM)

   Total volume of resuspension reaction: 13.5 μl.
10. Incubate at 65 °C for 5 min and quickly chill on ice for 2 min.
11. Add the following:

    4 μl 5× First strand reverse transcriptase buffer

    1 μl DTT(0.1 M)

    0.5 μl RNaseOUT (40 U/μl)

    1 μl Superscript III reverse transcriptase (200 U/μl)

    Total volume of resuspension reaction: 20 μl.
12. Incubate at 42 °C for 10 min, then 50 °C for 40 min, 85 °C for 5 min, and then hold at 4 °C.
13. Add 1 μl RNase A (20 μg/μl), incubate at 37 °C for 20 min.
14. Add 80 μl TE, 1 μl glycogen (5 μg/μl), 10 μl 3 M sodium acetate, and 300 μl 100 % ethanol.
15. Incubate at −20 °C overnight.

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3.9 Gel Purification and Circularization of cDNA

1. Spin down at 15,000 × *g* for 15 min.
2. Wash pellet with 70 % ethanol, air dry the pellet and resuspend it in 5 μl 1× RNA gel-loading dye.
3. Heat sample at 75 °C for 2 min and load it on a 8 % Urea-PAGE gel along with a molecular weight marker. Cut out 80–300 nt (nucleotide) gel pieces and elute the cDNAs from the gel pieces with 400 μl TE at room temperature overnight.
4. Spin down at 15,000 × *g* for 1 min, take out the supernatant, and add 40 μl 3 M sodium acetate, 1 ml 100 % ethanol, and 1 μl glycogen (5 μg/μl).
5. Incubate overnight at −20 °C.
6. Spin down at 15,000 × *g* for 15 min, wash the pellet with 70 % ethanol, dissolve the pellet in 12 μl water and add:

   1.5 μl 10× CircLigase buffer II

   0.75 μl $MnCl_2$ (50 mM)

   0.75 μl CircLigase II (100 U/μl)

   Total volume of resuspension reaction: 15 μl.

   Incubate at 60 °C for 2 h.
7. Add: 26 μl water

   5 μl 10× Fastdigest buffer

   1 μl Annealing oligo (10 μM)

   Total volume of resuspension reaction: 50 μl.

   Heat at 95 °C at 2 min.
8. Slowly cool down to room temperature.
9. Add 3 μl FastDigest *Bam* HI (20 U/μl) and incubate at 37 °C for 30 min.
10. Add 50 μl TE, 1 μl glycogen (5 μg/μl), 10 μl 3 M sodium acetate, and 300 μl 100 % ethanol. Incubate overnight at −20 °C.

3.10 PCR Amplification

1. Spin down at 15,000 × *g* for 15 min, wash the pellet, and dissolve pellet in 36.5 μl water. Then add:

   1 μl dNTPs (10 mM each)

   1 μl P5Solexa (10 μM)

   1 μl P3Solexa (10 μM)

   10 μl 5× High-fidelity phusion polymerase buffer

   0.5 μl High-fidelity phusion polymerase (2 U/μl)

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Total volume of resuspension reaction: 50 μl.

2. Run the following PCR program on a thermocycler:
   a. 98 °C 30 s
   b. 98 °C 10 s
   c. 65 °C 30 s
   d. 72 °C 30 s
   e. Go to (b) for 29 cycles
   f. 72 °C for 5 min
   g. Hold at 4 °C.
3. Purify the PCR products using the PCR purification kit.
4. Run a 1 % agarose gel to examine the size. A typical gel picture of the iCLIP library is shown in Fig. 2.
5. Measure the DNA concentration. Check with your sequencing facility about the required DNA concentration (usually 20–100 nM) and adjust your sample accordingly.

3.11 Bioinfomatic Analysis

1. Raw reads are first demultiplexed using unique sequencing barcodes, and the random trinucleotides identifying individual cDNA molecules are clipped.
2. The remaining sequences are then filtered and mapped to the reference genome using bowtie with the setting (-n 2 -m 1 -s 1) (up to two nucleotide mismatches and one unique match to the reference genome allowed) [11].
3. After mapping, reads that truncate at the same sites and have the same barcodes are combined.
4. For each read, the base upstream of the 5′ end is marked as the crosslinking site, and the total number of reads sharing the same crosslinking site on the same strand, called “cDNA count” is calculated.
5. Crosslinking sites identified in multiple replicate libraries are considered high confidence sites and are used for further analyses.

For further details on the bioinformatics analysis, please refer to refs. 8, 10, 12.

4 Trouble-Shooting Tips

1. All the homemade solutions are prepared with Milli-Q water.
2. A Thermomixer or a similar shaker is recommended for iCLIP library construction.
3. UV crosslinking needs to be optimized. 100–400 mJ/cm^2 is generally recommended for most proteins.

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4. Highly specific IP is key to the success of iCLIP-seq analysis and should be carefully optimized. An example of the quality control experiments we performed for CstF64 is shown in Figs. 1 and 2. The top panel of Fig. 1 displays a phosphorimage following gel transfer to nitrocellulose membrane (Subheading 3.7). First, a strong smeary band was observed in the IP sample that extends upwards from the expected size of CstF64 (lane 2). This corresponds to the CstF64–RNA complexes. Second, when the cell lysate was treated with RNase I, a sharper band at the expected size of CstF64 appeared, which corresponds to CstF64 crosslinked to small RNA fragments (lane 1). Third, when UV irradiation was omitted (lanes 3–4) or when IP was performed using protein A beads alone (lanes 5–8), no protein–RNA complex signal was detected. Fourth, when CstF64 is knocked down by RNAi (Fig. 1, bottom panel), the protein–RNA complex signal was proportionally reduced (lanes 9–10). Finally, specific CLIP PCR products were only obtained in the IP sample and no specific PCR products were detected when no antibody was used (Fig. 2). These experiments demonstrate that the UV crosslinking and IP were efficient and specific. It is highly recommended that similar pilot experiments be carried out all CLIP analyses to ensure the specificity.
5. RNase digestion needs to be optimized. Several RNases have been used in CLIP analyses, including RNase A, RNase A/T1 mix, RNase I, and micrococcal nuclease (MN) [7, 8, 13, 14]. As MN activity is dependent on calcium, MN digestion can be terminated by using EGTA [13]. No matter which RNase is used, the amount of RNase and digestion time need to be carefully optimized to maximize the yield.
6. cDNA is purified on a 0.4 mm thick 8 % PAGE-urea gel. We usually cut out the band above the xylene cyanol which corresponds to ~80 nt. This step removes the free RT primers which may interfere with the following steps.
7. If the majority of the final PCR products are primer dimers (128 bp), there are two potential reasons. First, not enough protein–RNA complexes were IPed. In this case, the experiments should be further optimized or scaled up to increase the yield. Second, the RNAs or cDNAs may be lost. Carefully monitor the RNA in all steps. Following 5′ labeling, RNAs can be traced by using a Geiger counter.
8. Before submitting iCLIP libraries for high-throughput sequencing, it is recommended to clone an aliquot of the iCLIP-seq libraries into a DNA vector and sequence a few clones using Sanger sequencing. Carefully check the sequences to make sure the insert sizes are appropriate and the libraries are properly constructed.
9. As a final measure to ensure the specificity of iCLIP, it is recommended that three replicate libraries from individually processed samples be constructed and sequenced. Careful comparisons of the replicates provide valuable information on the reproducibility of the analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

This work was supported by an NIH grant R01GM090056 and ACS grant RSG-12-186.

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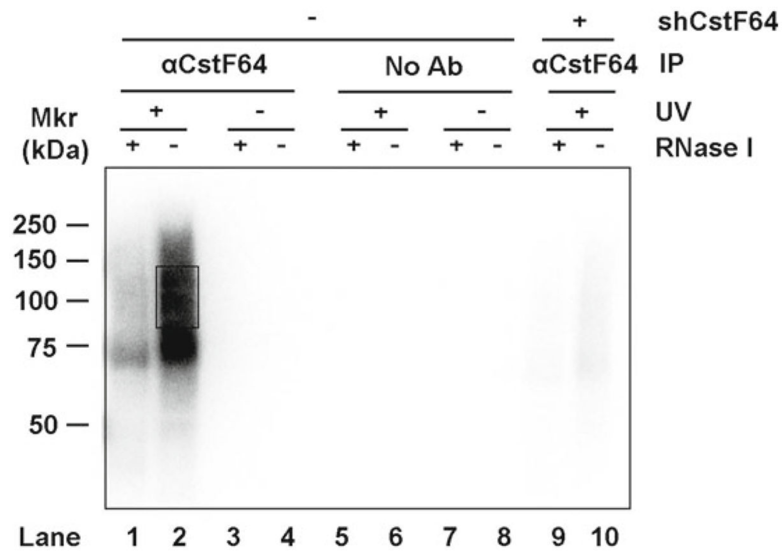

Fig. 1.

Top panel : Autoradiography image of the 5′ ^{32} P-labeled RNA–protein complexes from IP using no antibody (No Ab) or anti-CstF64 antibodies (αCstF64) with cell lysates from control HeLa (–shCstF64) or a HeLa cell line that stably expresses shRNAs targeting CstF64 mRNA (+shCstF64). The rectangle area was cut out from the membrane for subsequent steps. *Bottom panel* : CstF64 and actin western blotting results of the lysates used for IP in the *top panel*. (Gel images are from ref. 13)

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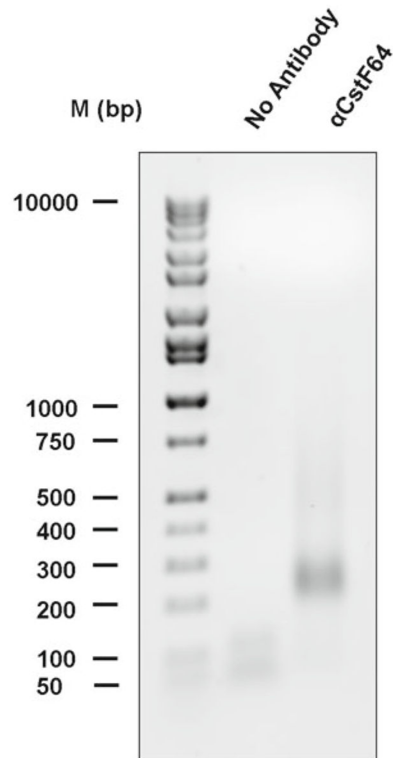

Fig. 2.

SYBR staining of PCR-amplified CstF64 iCLIP cDNA library (*lane 2*) and the no antibody negative control (*lane 1*). Sizes of the DNA ladder are marked