Chapter 19

RNA-Binding Proteins and Their Targets in *Trypanosoma brucei*: Single Nucleotide Resolution Using iCLIP and iCLAP

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Abstract

RNA-binding proteins (RBPs) are critical to posttranscriptional gene regulation. Therefore, characterization of the RNA molecules bound by RBPs in vivo represent a key step in elucidating their function. The recently developed iCLIP technique allows single nucleotide resolution of the RNA binding footprints of RBPs. We present the iCLIP technique modified for its application to *Trypanosoma brucei* and most likely other kinetoplastid flagellates. By using the immuno- or affinity purification approach, it was successfully applied to the analysis of several RBPs. Furthermore, we also provide a detailed description of the iCLIP/iCLAP protocol that shall be particularly suitable for the studies of trypanosome RBPs.

Key words RNA-binding proteins (RBPs), iCLIP, iCLAP, Posttranscriptional gene regulation

1 Introduction

Posttranscriptional gene regulation is critical for the maintenance and control of gene expression levels [1]. Interestingly, *Trypanosoma brucei* lacks regulation at the transcriptional level, which means that it has to rely extensively on posttranscriptional mechanisms [2]. Numerous RNA-binding proteins (RBPs) are known to be involved in posttranscriptional gene regulation [3]. To elucidate the role of a given RBP, it is important to characterize its RNA targets in vivo. However, initially developed approaches such as RIP-sequencing lack sufficient resolution and specificity to identify binding sites of the studied RBPs [4]. The relatively recently established in vivo UV cross-linking and immunoprecipitation (CLIP) technique provides high specificity and single nucleotide resolution [5]. Still, a significant drawback of the CLIP protocol is the use of 3′ and 5′ adapters during library preparation. This feature makes the CLIP protocol insufficient in capturing truncated cDNAs at the reverse transcriptase step. Hence, a CLIP variant called individual-nucleotide resolution CLIP (iCLIP) has been developed, which

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uses 3′ adapters and incorporates a circularization step that allows an efficient capture of truncated cDNAs [6].

Most of the abovementioned techniques were developed in cells that lacked flagella and consequently were immotile. We have successfully applied the iCLIP technique and its variant that uses a two-step-based affinity purification (iCLAP) to the study of three RBPs that participate in the uridine-insertion/deletion type of RNA editing in the mitochondrion of *T. brucei* [7, 8]. While our protocol for the iCLIP library preparation remains generally the same as the original iCLIP protocol developed previously [9], some modifications have been made. Hence, we provide the iCLIP or iCLAP protocols that can be easily applied to the studies of RBPs in *T. brucei* and other kinetoplastid flagellates.

2 Materials

2.1 iCLIP Buffers

1. iCLIP lysis buffer: 100 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% SDS, 1% NP-40, 1× protease inhibitor (freshly prepared).
2. iCLIP high-salt wash buffer: 100 mM Tris–HCl (pH 7.5), 1 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS.
3. PNK buffer: 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.2% Tween 20.
4. PK buffer: 100 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM EDTA.

2.2 iCLAP Buffers

1. iCLAP lysis buffer: 50 mM Tris–HCl (pH 7.5), 1.5 mM $MgCl_2$, 10% glycerol, 250 mM NaCl, 0.5% NP-40, 0.1% SDS, 2.5 mM beta-mercaptoethanol (freshly prepared), 1× protease inhibitor (freshly prepared).
2. iCLAP wash buffer: 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 0.1% NP-40, 2.5 mM beta-mercaptoethanol.
3. TEV cleavage buffer: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% NP-40, 2.5 mM beta-mercaptoethanol.
4. His-binding buffer: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% NP-40, 10 mM imidazole.
5. Urea buffer: 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.1% NP-40, 10 mM imidazole, 7 M urea.
6. PNK buffer: 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.2% Tween 20.
7. PK buffer: 100 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA.

2.3 UV-Cross Linking

Stratalinker UV cross-linker 2400, Protease inhibitor cocktail, IgG Sepharose beads, Anti-RNase, RNAse I, Turbo DNase, T4 PNK plus 10× PNK buffer, RNasin, Protein spin columns, T4 RNA Ligase I, γ-^{32}P-ATP, Phosphate buffered saline (PBS), Falcon tubes, Shrimp alkaline phosphatase, Protein G Dynabeads, IgG Sepharose 6 fast flow affinity resin, His-Tag Isolation Dynabeads.

2.4 SDS-PAGE and Nitrocellulose Transfer

4–12% NuPAGE gels, electrophoresis chamber, transfer apparatus (Life Technologies), LDS-4X sample buffer, prestained protein marker, nitrocellulose membrane, sponge pads for XCell II blotting, 20× transfer buffer, 20× MOPS-SDS running buffer, Whatman filter paper GE Healthcare, Film (Fuji).

2.5 RNA Isolation

Proteinase K, 19 G syringe needles, phenol and chloroform, phase lock gel heavy tube (VWR), glycogen, 3 M sodium acetate (pH 5.5).

2.6 Reverse Transcription

PCR tubes, dNTPs, Superscript III (Life Technologies), 5× First-strand buffer (Life Technologies), dithiothreitol, 1 M HEPES, TE buffer.

2.7 cDNA Isolation and PCR Amplification

2× TBE-urea loading buffer (Life Technologies), 6% TBE-urea precast gels (Life Technologies), low molecular weight marker, TBE running buffer, SYBR Green II (Life Technologies), 19 G syringe needle, glass prefilters (Whatman), phase lock gel heavy (VWR), Costar SpinX column (Corning Incorporation), 10× CircLigase buffer, CircLigase II (Cambio), $MnCl_2$, BamHI (Fermentas), Fast digest buffer (Fermentas), Accurprime Supermix I (Life Technologies), SYBR Green I (Life Technologies).

2.8 Trypanosome Culture

Procyclic form of *T. brucei* strain Lister 427 29-13 cell line was cultured using SDM79 with appropriate selection drugs as previously mentioned [7, 8].

3 Methods

3.1 UV Cross-Linking of T. brucei (Fig. 1): Day 0

1. Grow trypanosomes in SDM79 medium with appropriate selection by drugs to a density of 2×10^7 cells/ml.
2. Use the culture from **step 1** as a starting culture to scale up to 1000 ml. If required, add tetracycline to express protein of interest (*see* **Note 1**).
3. Harvest the cells once they reach a density of 2×10^7 cells/ml, by spinning them down at 2600 rpm (~950 rcf) for 10 min.
4. Resuspend the pellet in 20 ml of cold PBS and split it into 2×10 ml in two 15 ml Falcon tubes.

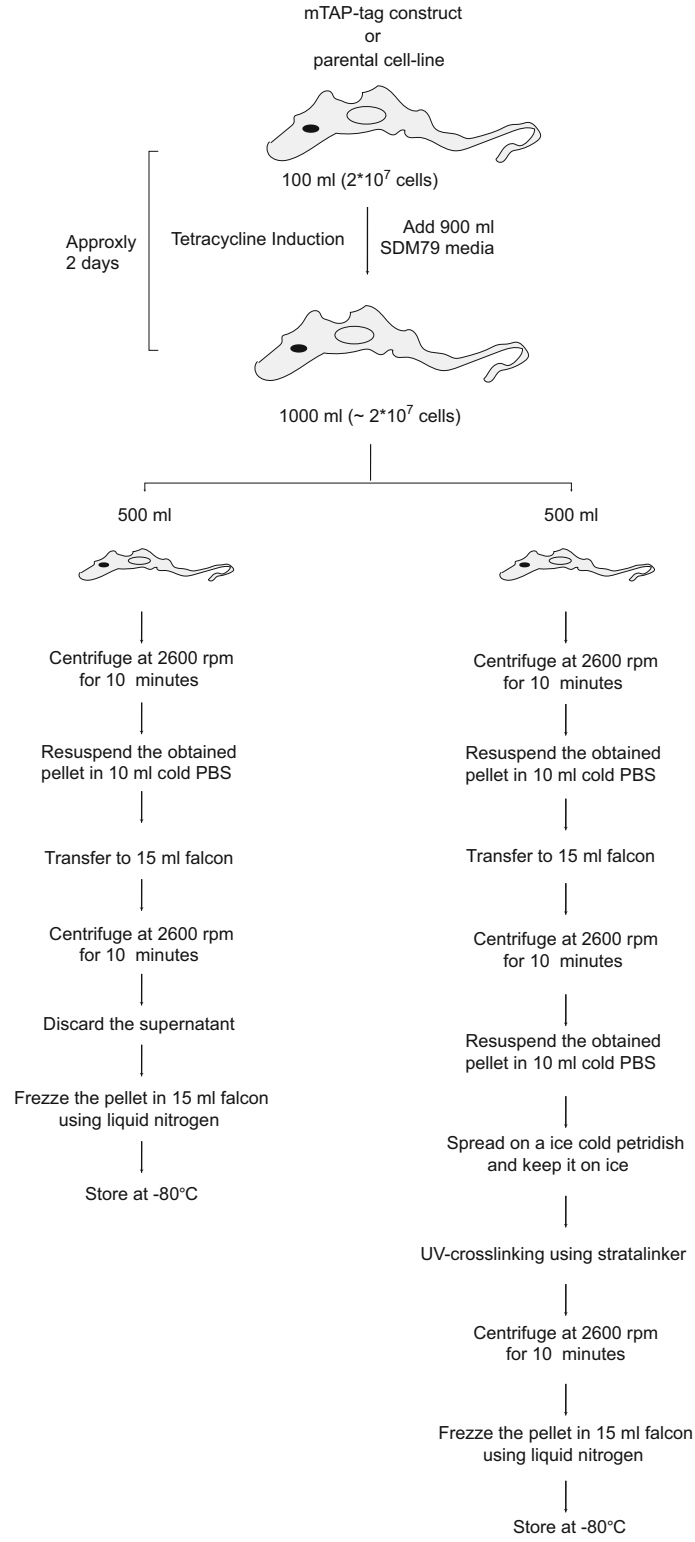

Fig. 1 Schematic depiction of the in vivo UV cross-linking protocol in trypanosomes

5. Spin both Falcon tubes at 2600 rpm (~950 rcf) for 10 min and carefully discard the supernatants.
6. Freeze one pellet in liquid nitrogen and keep it at −80 °C until use in the iCLIP experiment. Label the tube as non-UV-cross-linked pellet that is required as a negative control for the subsequent iCLIP protocol (*see* **Note 2**).
7. Resuspend the second pellet in 10 ml cold PBS and use it for UV cross-linking.
8. Spread the trypanosomes from **step 7** onto a prechilled petri dish (140 mm diameter) and keep them on ice.
9. Take the petri dish along with ice tray from **step 8** and UV irradiate trypanosomes in the Stratalinker.
10. Transfer the UV irradiated cells from the petri dish to a 15 ml Falcon tube and spin them at 2600 rpm for 10 min (*see* **Note 3**).
11. Carefully discard the supernatant and freeze the tube in liquid nitrogen. Label it as UV-cross-linked pellet and keep it at −80 °C until its use in the iCLIP experiment (*see* **Note 4**).

3.2 iCLIP (In Vivo Cross-Linking and Immunoprecipitation): Day 1 (Fig. 2)

3.2.1 Lysate Preparation

1. Thaw the UV- and non-UV-cross-linked pellets on ice (~15 min) and resuspend them in 1.5 ml cold lysis buffer, which is freshly supplemented with protease inhibitor (*see* **Note 5**).
2. Clean the sonicator tip with RNAseZap and then with milliQ water.
3. Sonicate both samples (5–10 pulses each 30 s at 50% amplitude with 20 s pause in-between) (*see* **Note 6**).
4. Transfer the lysate to a fresh 1.5 ml microcentrifuge tube and spin it for 10 min at maximum speed of 16,110 × *g* at 4 °C in a benchtop centrifuge (*see* **Note 7**).
5. Transfer the supernatant into a fresh tube (leave some to prevent carryover). We usually get enough supernatant that is diluted and used to carry out simultaneously several control immunoprecipitation assays (*see* **Note 8**).

3.2.2 Partial RNA Digestion

1. Prepare a dilution of RNAse I at 1:500 (low RNase treatment) and 1:50 (high RNase treatment) in cold lysis buffer under the hood (*see* **Note 8**).
2. Add 10 μl of low RNase I dilution (1:500) and 2 μl of Turbo DNase per 1.5 ml of freshly prepared lysate (from **step 5**, Subheading 3.2.1) (*see* **Note 9**).
3. Incubate the lysate at 37 °C for 3 min while shaking at 800 rpm in a thermomixer.
4. Place the reaction on ice for 3 min (*see* **Note 10**).

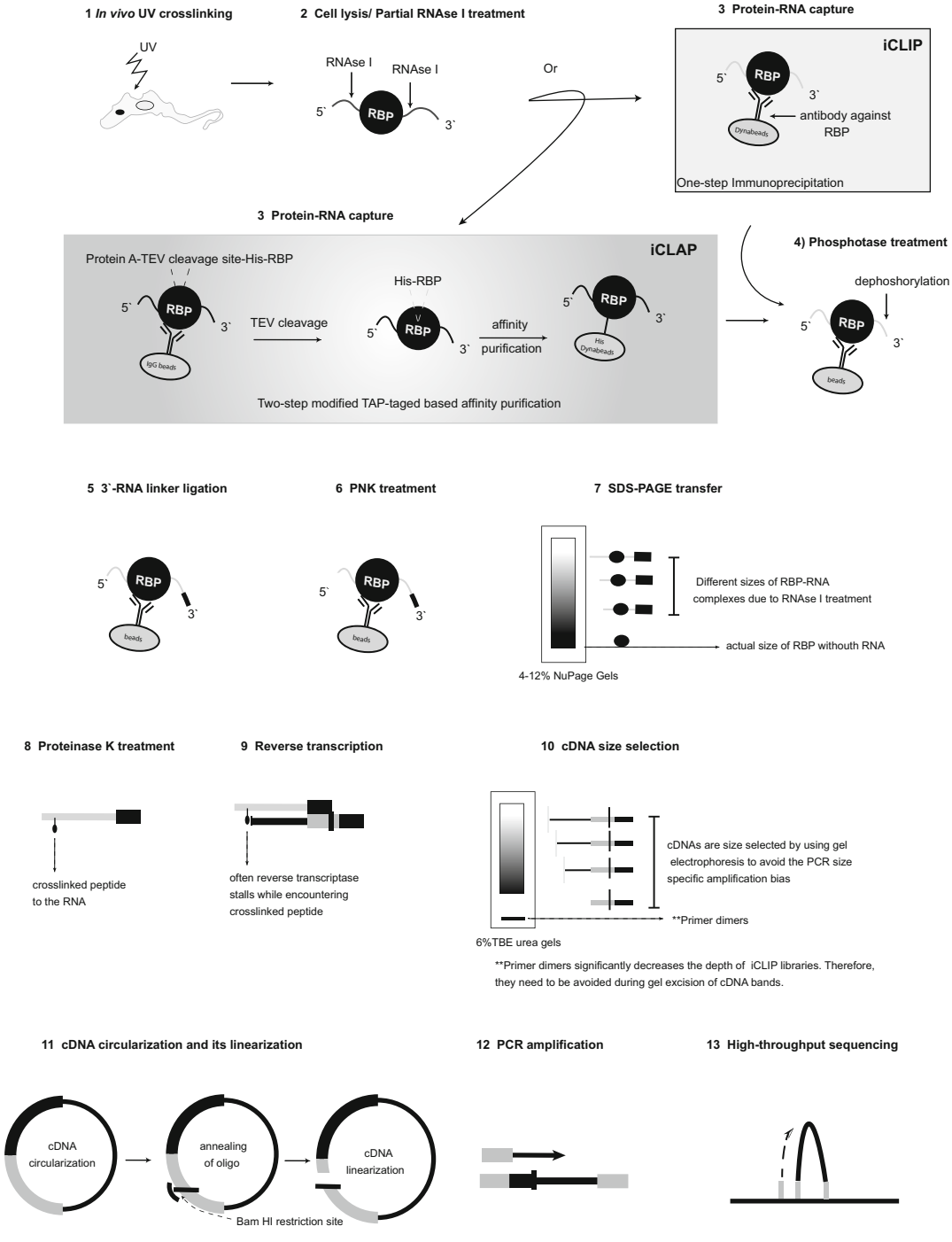

Fig. 2 Preparation of the iCLIP or iCLAP libraries

3.2.3 Bead Preparation

1. Rotate the protein G dynabeads, that come in the form of a suspension, for 5 min on a rotator. Take 100 μl of resuspended protein G dynabeads in the 1.5 ml microcentrifuge tube per immunoprecipitation (IP) assay (*see* **Note 11**).
2. Stack the microcentrifuge tube containing protein G dynabeads into the magnetic rack for 45 s (usually it takes ~45 s for the beads to settle close to the magnetic side of the microcentrifuge tube) and carefully pipette out all the residual buffer.
3. Wash the protein G dynabeads 3× with 500 μl cold lysis buffer (each wash for 5 min at 4 °C).
4. Resuspend protein G dynabeads into 200 μl cold lysis buffer and add an already optimized amount of the antibody (*see* **Note 12**).
5. Let the tube rotate for 45 min at 4 °C.
6. Wash the antibody-coupled protein G dynabeads 3× for 5 min in cold lysis buffer at 4 °C.
7. The protein G dynabeads are now ready for immunoprecipitation. Keep them on ice until you are ready to proceed to the next step.

3.2.4 Immunoprecipitation

1. Add 1.5 ml of lysate (from **step 4** of partial RNA digestion, Subheading 3.2.2) to antibody-coupled protein G dynabeads (from **step 7** of bead preparation, Subheading 3.2.3).
2. Incubate the dynabeads on a rotator for 1 h at 4 °C.
3. Use the magnetic rack to discard the supernatant from the dynabeads.
4. Wash the dynabeads 2× with 500 μl of high-salt wash buffer for 2 min each at 4 °C.
5. Wash the dynabeads 2× with 500 μl of PNK buffer for 2 min each at 4 °C.
6. Resuspend the dynabeads in 1 ml of PNK buffer.
7. Transfer 100 μl of resuspended dynabeads into a new microcentrifuge tube and leave it at 4 °C overnight. It will be used next day for the 5′ radiolabeling of RNA (*see* Subheading 3.4 and also **Note 13**).
8. For each sample prepare the 3′ RNA dephosphorylation master mix:

   8 μl water.

   1 μl of 10× Shrimp alkaline phosphatase (SAP) buffer.

   1 μl of Shrimp alkaline phosphatase (SAP).

   (*See* **Note 14**).

9. Remove the supernatant from the remaining 900 μl of dynabeads and resuspend the beads in 10 μl of 3′ RNA dephosphorylation master mix.
10. Incubate the reaction at 37 °C for 20 min.
11. Wash the dynabeads on a rotator 2× with 500 μl of high-salt wash buffer for 2 min each at 4 °C (*see* **Note 15**).
12. Wash them on a rotator 2× with 500 μl of PNK buffer for 2 min each at 4 °C.
13. Prepare the 3′ RNA linker ligation mix per sample:

    3.5 μl water.

    0.75 μl 10× RNA ligase buffer.

    2.5 μl PEG 400.

    0.25 μl T4 RNA ligase 1.

    0.1 μl RNase OUT.

    3 μl linker L3.
14. Remove the supernatant from **step 12** and resuspend the beads in 10 μl of the 3′ RNA linker ligation mix.
15. Place the microcentrifuge tube overnight at 16 °C for linker ligation. After this step go directly to Subheading 3.4.

3.3 iCLAP Protocol (In Vivo Cross-Linking and Affinity Purification Protocol)

This is a separate protocol that uses a modified TAP-tag (protein of interest with His_6-TEV protease cleavage site-protein A) to purify a given protein–RNA complex from trypanosomes. It uses a two-step affinity purification, while the downstream cDNA library preparation remains the same as the one used in iCLIP.

3.3.1 Preparation of the Lysate: Day 1 (Fig. 2)

1. Thaw UV- and non-UV-cross-linked pellets on ice (~15 min) and resuspend them in 1.5 ml cold iCLAP lysis buffer, which is freshly supplemented with the protease inhibitor (*see* **Note 5**).
2. Clean the sonicator tip with RNAseZap and then with milliQ water.
3. Sonicate both samples (five pulses each 30 s at 50% amplitude, with 20 s pause in-between) (*see* **Note 6**).
4. Transfer 1.5 ml lysate to a fresh 1.5 ml microcentrifuge tube and spin for 10 min at maximum speed of 16,000 × *g* at 4 °C (*see* **Note 7**).
5. Transfer the supernatant to a fresh 1.5 ml microcentrifuge tube (leave some supernatant to prevent carryover).

3.3.2 Partial RNA Digestion

Same as partial RNA digestion in Subheading 3.2.2.

3.3.3 Preparation of IgG Sepharose Beads

1. Add 300 µl of IgG Sepharose beads into a 1.5 ml microcentrifuge tube and spin it at 200 × *g* for 30 s. Keep the microcentrifuge tube on ice and let the IgG beads settle for ~45–60 s. Once the IgG beads are settled, discard the supernatant (*see* **Note 16**).
2. Add 1 ml of cold iCLAP lysis buffer to the IgG beads and let it spin on a rotator for 5 min at 4 °C. Spin the microcentrifuge tube at 1200 rpm for 30 s, and afterward keep the tube on ice for 45 s. Once beads are fully settled, discard the supernatant.
3. Wash the IgG beads 3× with the iCLAP lysis buffer.
4. Keep the equilibrated IgG beads on ice until the lysate is ready.

3.3.4 IgG Sepharose-Based Affinity Purification

1. Once the lysates are ready, dilute them in the iCLAP lysis buffer by bringing the total volume to 10 ml and use a fresh 15 ml Falcon tube to load both equilibrated IgG beads and diluted lysis buffer.
2. Let them incubate for 2 h at 4 °C on a rotator.
3. Wash IgG beads in wash buffer 3× for 5 min each.
4. Resuspend IgG beads in 1 ml of TEV cleavage buffer and transfer it to a 1.5 ml tube.
5. Wash the IgG beads in 1 ml TEV cleavage buffer 3× for 5 min each.
6. After the last wash, resuspend the IgG beads in 100 µl of TEV cleavage buffer and 20 µl of homemade GST-TEV protease (depending upon the batch but usually ~20 U) (*see* **Note 17**).
7. For TEV protease cleavage, keep the tube in a thermomixer at 18 °C while shaking at 1000 rpm.
8. After 2 h, transfer the beads along with the supernatant to a 1.5 ml protein purification column and spin the column at 250 × *g* for 1 min (*see* **Note 18**).
9. Collect the flow-through into a separate 1.5 ml tube.
10. Add 1.2 ml of His-binding buffer to a flow-through and keep it on ice until His-dynabeads are ready.

3.3.5 Preparation of His-Dynabeads

1. Transfer 100 µl of the His-dynabeads into a fresh 1.5 ml tube and remove the supernatant from the beads using a magnetic rack (*see* **Note 19**).
2. Wash the His-dynabeads on a rotator with 1 ml of His-binding buffer 3× for 2 min at 4 °C.

3.3.6 His Dynabeads-Based Affinity Purification

1. Resuspend the collected flow-through from **step 10** (Subheading 3.3.4) to already equilibrated His-dynabeads from **step 2** (Subheading 3.3.5).
2. Let them incubate on a rotator for 1 h at 4 °C.

3. Wash the His-dynabeads on a rotator with 500 µl of urea wash buffer 2× for 5 min each at 4 °C.
4. Wash the His-dynabeads on a rotator with 1 ml of PNK buffer 2× for 2 min each at 4 °C.
5. Resuspend the His-dynabeads in 1 ml PNK buffer.
6. Transfer 100 µl of resuspended His-dynabeads into a new tube and leave it at 4 °C overnight (will be used the next day for 5′ radiolabeling).
7. Prepare 3′ RNA dephosphorylation master mix per sample:

   8 µl nuclease-free water.

   1 µl of 10× shrimp alkaline phosphatase (SAP) buffer.

   1 µl of shrimp alkaline phosphatase (SAP).
8. Remove the supernatant from the remaining 900 µl of the His-dynabeads and resuspend the beads in 10 µl of 3′ RNA dephosphorylation master mix.
9. Incubate the reaction at 37 °C for 20 min.
10. Wash the His-dynabeads on a rotator with 500 µl of high-salt wash buffer 2× for 2 min each at 4 °C.
11. Remove the supernatant and on a rotator wash the His-dynabeads with 500 µl PNK buffer 2× for 2 min each at 4 °C.
12. Next step is a 3′-linker ligation of the RNA. Prepare a L3 linker ligation mix per sample:

    0.75 µl 10× RNA ligase buffer.

    3.5 µl water.

    2.5 µl PEG 400.

    0.25 µl T4 RNA ligase 1.

    0.1 µl RNase OUT.

    3 µl linker L3.
13. Remove the PNK buffer from the His-dynabeads (**step 11**) and resuspend them in 10 µl of the L3 linker ligation mix.
14. Keep the tube overnight at 16 °C.

3.4 SDS-PAGE and Transfer of Protein–RNA Complexes to Nitrocellulose Membrane: Day 2

From here on, all subsequent steps are the same for both the iCLIP and iCLAP protocols. In case you want to carry out the iCLIP protocol, go directly from Subheading 3.2.4 to this subheading, or in case of the iCLAP protocol, go directly from Subheading 3.3.6 to this subheading.

1. Next day prepare a hot PNK master mix for 5′ radiolabeling of the RNA per sample:

   0.2 µl T4 PNK.

0.4 μl 10× PNK buffer.

0.4 μl γ-^{32}P-ATP.

2 μl nuclease-free water.

2. For iCLIP: remove 100 μl of the protein G dynabeads from **step 7** (Subheading 3.2.4), discard the supernatant using the magnetic rack and resuspend them in the hot PNK master mix. While, for iCLAP: remove 100 μl of the His-dynabeads from **step 6** (Subheading 3.3.6), discard the supernatant and resuspend them in the hot PNK master mix. In both cases mix the samples well and incubate them at 37 °C for 10 min (keep in mind that the beads are now radioactively hot).
3. Dilute 4× NuPage protein loading dye to 1.5× and prepare a 10-well 4–12% NuPage Bis-Tris gel with 1× MOPS running buffer.
4. For iCLIP: remove 900 μl of the protein G dynabeads from **step 15** (Subheading 3.2.4) and discard the linker ligation mix.

   For iCLAP: remove 900 μl of the His-dynabeads from **step 14** (Subheading 3.3.6) and discard the linker ligation mix.

   Wash the dynabeads once with 500 μl of PNK wash buffer for 2 min at 4 °C. These beads are radioactively cold and contain 3′ RNA linkers.
5. Discard the PNK wash buffer from the above step and resuspend the cold dynabeads into 25 μl of 1.5× NuPage loading dye.
6. After 10 min incubation discard the hot PNK mix from the hot dynabeads.
7. Pool the radioactively cold and hot dynabeads together using 25 μl of 1.5× NuPage loading dye.
8. Heat the dynabeads for 10 min at 70 °C.
9. Collect 25 μl of 1.5× NuPage supernatant from the dynabeads using the magnetic rack.
10. Load the supernatant directly into one well of the 4–12% NuPage Bis-Tris gel. In case of more samples leave one lane free between the samples to decrease the chances of RNA cross contamination.
11. Load the prestained marker for the reference in the last well and run the gel for 45 min at 180 V.
12. After the run is finished, cut the bottom of the gel that contains free ATP and discard it in a solid radioactive waste bin.
13. Transfer the remaining gel onto a nitrocellulose membrane using a Novex wet transfer apparatus and Novex transfer buffer supplemented with 20% methanol. The condition used for transfer is 2 h at 45 V and 4 °C.

14. Once the transfer is finished, rinse the membrane in sterile cold PBS and wrap it with Saran wrap.
15. Transfer the membrane into a cassette and expose the film at −80 °C. Analyze the exposure at three different time points (suggested times are 30 min, 1 h, and overnight).

3.5 RNA Isolation: Day 3

1. Analyze the overnight exposure and place the developed autoradiogram precisely over the nitrocellulose membrane.
2. Use the autoradiogram as a reference to mark the square that needs to be cut out of the nitrocellulose membrane with a marker that corresponds to the protein–RNA complex of your interest (*see* **Note 20**).
3. Take the marked nitrocellulose membrane out of the cassette and use a fresh scalpel to cut the marked area into tiny pieces.
4. Place the tiny nitrocellulose membrane pieces into a fresh 1.5 ml tube and add to it 200 μl of PK buffer.
5. Add 10 μl of proteinase K into the tube and incubate it in a thermomixer at 1000 rpm for 10 min at 37 °C.
6. After 10 min incubation add 200 μl of PK buffer that contains 7 M urea and incubate the tube for another 20 min at 37 °C.
7. Transfer the supernatant carefully without taking the membrane pieces from the microcentrifuge tube to a 2 ml phase lock gel tube.
8. Add 400 μl acidic phenol and chloroform–isoamyl alcohol to the 2 ml phase lock gel tube and incubate the reaction at 30 °C while shaking at 1000 rpm for 5 min (do not vortex).
9. After 5 min spin the phase lock gel tube at maximum speed in a benchtop centrifuge.
10. Carefully transfer the upper aqueous layer to a fresh 1.5 ml tube (leave some to avoid the carryover) and add to it 1 μg of glycogen, 50 μl of 3 M sodium acetate (pH 5.5), and 1 ml of 100% ethanol.
11. Precipitate RNA overnight at −20 °C (alternatively for 1 h at −80 °C).

3.6 Reverse Transcriptase: Day 4

1. Take out the tube with the precipitated RNA and spin it for 30 min at 13,000 rpm and 4 °C.
2. Carefully remove the supernatant without disturbing the pellet, add 500 μl of 70% ethanol and spin the samples for 15 min at 13,000 rpm and 4 °C.
3. Carefully remove the supernatant without disturbing the pellet and let the pellet dry for 5–10 min at room temperature.
4. Once the pellet is dry, resuspend the (usually invisible) pellet in 6 μl of nuclease-free water.

5. Dilute RCLIP primers to 0.1 μM (mix 2.5 μl water and 0.5 μl primer from 2 μM stock) (*see* **Note 21**).
6. Prepare dNTP–primer mix in a PCR tube (include one tube for reverse transcriptase (RT)-control in 6 μl of nuclease-free water):
   0.5 μl RPCLIP primer specific for L3 linker (0.1 pmol/μl; from **step 5**).
   0.5 μl dNTP Mix (10 mM each).
   (*See* **Note 22**).
7. Transfer 6 μl of RNA (from **step 4**) to the already prepared dNTP–primer mix in a PCR tube (from **step 6**).
8. Place the PCR tube in the PCR cycler machine and start the RT thermal program:
   *70 °C for 5 min.
   Hold at **25 °C until the RT mix is added.
   42 °C for 20 min.
   50 °C for 40 min.
   4 °C hold.
   While the samples are being denatured at *70 °C for 5 min, prepare a master mix for the RT reaction (including RT control) per sample:
   2 μl 5× RT buffer.
   0.5 μl DTT (0.1 M).
   0.25 μl Superscript III RT (200 U/μl).
   Once the temperature reaches **25 °C, add 2.75 μl of freshly prepared RT master mix to each PCR tube and mix well.
9. Take the tubes out from the cycler, add 90 μl of TE buffer, transfer it to new 1.5 ml tube, add 0.5 μl glycogen and 15 μl sodium acetate and mix well.
10. Add 300 μl of ethanol, mix well and precipitate the RNA overnight at −20 °C (alternatively for 1 h at −80 °C).

3.7 Size Selection of cDNA: Day 5

1. Take the tube with the overnight precipitated cDNA out and spin it for 30 min at 13,200 rpm and 4 °C.
2. Carefully remove the supernatant without disturbing the pellet, add 500 μl of 70% ethanol and spin the samples for 15 min at 13,200 rpm and 4 °C.
3. Carefully remove the supernatant without disturbing the pellet, let the pellet dry for 10 min at room temperature, and resuspend it in 6 μl of nuclease-free water.
4. To 6 μl of this cDNA add another 6 μl of 2× TBE-urea loading buffer.

5. Heat the samples for 3 min at 70 °C.
6. Place a precasted 6% of TBE-urea gel in a gel running chamber and fill it with 1× TBE running buffer.
7. Load 12 μl of cDNA (from **step 5**) using a long pipette tip. It is important to flush the urea from the wells using a needle just before loading of the cDNA. Always leave one lane free between the samples to avoid possible cross contamination. Also load a DNA size marker.
8. Run the gel at 180 V for exactly 40 min.
9. After the run is finished, cut the last lane with the DNA size marker and incubate it for 15 min in 25 ml TBE buffer with 1 μl of SYBR Green II. Take a scan for a DNA size marker using ChemiDoc and print the result by making sure it is in size exactly to 100% scale.
10. Use the printed DNA size marker result from the above step as a reference to excise the size selected cDNA from the TBE-urea gel.
11. Cut out bands from the gel in 3 sizes of approximately 60–90, 90–120, and 120–200 nucleotides and place them in three different 1.5 ml tubes (*see* **Note 23**).
12. Add 400 μl of TE buffer to each tube and crush the gel into fine pieces using the plunger from a 1 ml syringe.
13. Incubate the gel pieces in the tube at 37 °C for 2 h in a thermomixer while shaking at 200 × *g*.
14. Add 1 cm of glass prefilter to a Costar SpinX column and transfer it into a fresh 1.5 ml tube.
15. Transfer the supernatant from the tube containing gel pieces onto the Costar SpinX column.
16. Spin the Costar tube for 2 min at maximum speed at room temperature.
17. Keep the flow-through (usually ~350 μl) and add 40 μl of sodium acetate, 0.5 μl glycogen, and 1 ml of 100% ethanol.
18. Precipitate the RNA overnight at −20 °C (alternatively for 1 h at −80 °C).

3.8 cDNA Circulation and Linearization: Day 6

1. Take the tube with the overnight precipitated cDNA out and spin it for 30 min at 13,200 rpm and 4 °C.
2. Carefully remove the supernatant without disturbing the pellet, add 500 μl of 70% ethanol and spin the samples for 15 min at 13,200 rpm and 4 °C.
3. Carefully remove the supernatant without disturbing the pellet and let it dry for 10 min at room temperature.

4. While the pellet is left to dry, prepare the cDNA circularization ligation mix:

   6.5 μl nuclease-free water.

   0.8 μl CircLigase buffer II.

   0.4 μl 50 mM $MnCl_2$.

   0.3 μl Circligase II.

5. Resuspend the dry pellet in 8 μl of ligation mix and transfer it to a fresh PCR tube.
6. Incubate the PCR tube for 1 h at 60 °C in PCR thermocycler, which is then set up for 4 °C on hold.
7. During the 4 °C on hold, prepare the oligo annealing mix:

   26 μl nuclease-free water.

   3 μl Fast digest buffer.

   1 μl of 10 mM CutC4 (complimentary DNA for BamHI restriction in next step).

8. Change the thermocycler settings to:

   95 °C for 2 min.

   70 cycles starting with 95 °C for 1 min, and decreasing the temperature by 1 °C in every cycle with the final temperature being 25 °C.

   25 °C on hold.

9. Add 2 μl of BamHI to the PCR tubes and change the thermocycler settings to 37 °C for 40 min (*see* **Note 24**).
10. Following the restriction digestion, add 60 μl of TE buffer and transfer the samples to a fresh 1.5 ml tube, to which add 0.5 μl of glycogen, 15 μl sodium acetate, and 300 μl 100% ethanol and mix everything properly.
11. Precipitate RNA overnight at −20 °C (alternatively for 1 h at −80 °C).

3.9 PCR Amplification: Day 7

1. Take out the tube with the overnight precipitated cDNA and spin it for 30 min at 13,000 rpm and 4 °C.
2. Carefully remove the supernatant without disturbing the pellet, add 500 μl of ice cold 70% ethanol and spin the samples for 15 min at 13,000 rpm and 4 °C.
3. Carefully remove the supernatant and let the pellet dry for about 10 min at room temperature.
4. Resuspend the dry pellet in 11 μl of nuclease-free water.
5. Prepare a PCR master mix in a PCR tube as follows:

   1 μl of cDNA (from **step 4**).

   9 μl of nuclease-free water.

   0.5 μl of P5/P3 Solexa primer mix (10 μM each).

6. Place the PCR tube into the PCR thermocycler with the following settings:

   95 °C for 10 min.

   95 °C for 10 s, 65 °C for 30 s, 68 °C for 30 s (20–25 cycles).

   72 °C 3 min.

   25 °C on hold.

7. Resolve the PCR products on a 1% agarose gel or 6% TBE-urea gel by loading 7 μl of the cDNA and stain it using 3 μl SYBR Green I for 10 min in 30 ml of 1× TBE.
8. Depending on the results, pool the PCR products from different size fractions into a single microcentrifuge tube (*see* **Note 25**).

4 Notes

1. While scaling up to a larger volume, you can skip adding the drugs to reduce the costs.
2. This is an important negative control which shall be done for every new RNA binding protein. This will allow the visualization of the background levels of pulled-down RNA with the target protein in the absence of UV cross-linking.
3. Since the UV treatment might lead to UV-induced activation of the DNA damage repair pathway, spin and freeze the cells in liquid nitrogen after UV cross-linking as quickly as possible. For successful UV cross-linking keep the Petri dish about 5 cm from the UV lamp in the Stratalinker.
4. Use the frozen pellets to carry out the iCLIP or iCLAP experiments within a month.
5. Use freshly made buffers which have been filtered through 0.22-μm filters.
6. Avoid foaming and ensure that during the process, the samples remain cold. Depending on the machine used, the duration of sonication and the amplitude have to be optimized before the initial experiment.
7. It is critical to optimize the lysate concentration used for the immunoprecipitation assay. We use 5 and 10 mg/ml of protein for the iCLIP and iCLAP experiments, respectively.
8. The high RNase treatment of the lysate is required only during the initial set up of the iCLIP assay and serves two purposes (*see* Fig. 3a). Firstly, it is used as a positive control to determine the specificity of the pull down of the protein–RNA complexes. Due to the high RNase treatment, it makes RNA to appear as a sharp band (usually ~5 kDa above the molecular weight of the

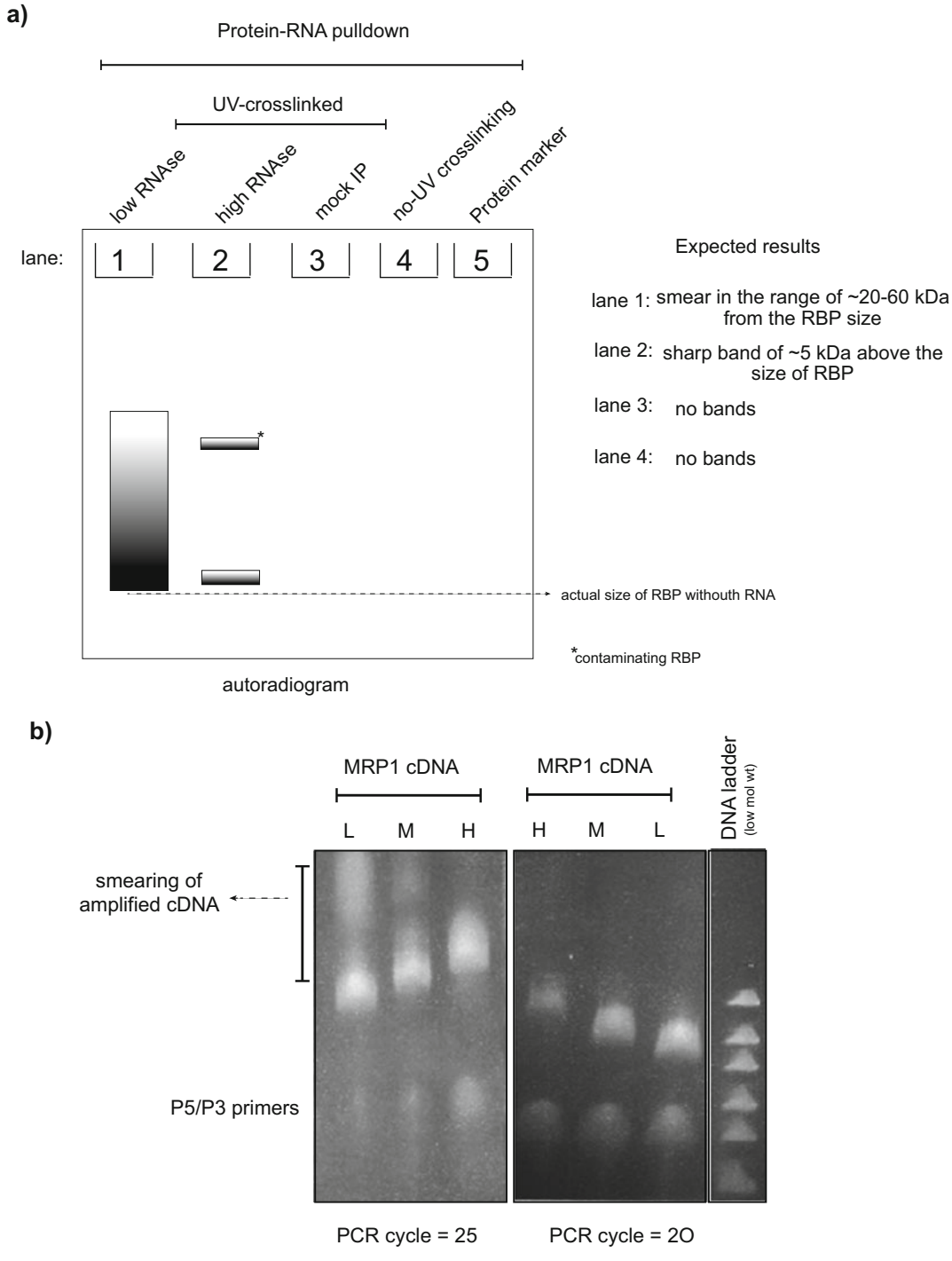

Fig. 3 Key steps in the iCLIP/iCLAP analysis. (**a**) Analysis of copurified RNA–protein complexes. The autoradiogram depicts the expected results and necessary controls. Low RNase I (lane 1) treated RNA–protein complex usually results in a smear and is used for preparation of the cDNA library. The high RNase I (lane 2) treated RNA–protein complex yields a sharp band. Mock immunoprecipitation (lane 3) and non-UV-cross-

protein of interest) in the autoradiogram as compared to a smear in case of low RNase-treated lysate. Therefore, if you obtain more than one radiolabeled RNA band in the autoradiogram, it possibly reflects the presence of another contaminating RBP. This will likely demand optimization of the protocol to obtain higher stringency in the pull-down experiments. Otherwise, if the contaminating RBP(s) have a considerable size difference with the RBP of interest, then you can still excise the target band from the nitrocellulose membrane. At this point you must carry out the other control immunoprecipitation (IP) assays to verify the stringency of IPs, which include non-UV-cross-linked and mock IPs with high RNase treatment. In both cases, this should not yield a radiolabeled RNA band. If all the controls work properly, proceed with the iCLIP library preparation protocol. Lastly, the use of RNase I is recommended given it has no base preference for its activity.

9. After all control IPs have been performed, you shall perform the iCLIP protocol for final library preparation. It requires low RNase treatment of the lysates and optimization for every new batch of RNase I. It is suggested that you test several low RNAse treatment conditions (1:250, 1:500, 1:800) (*see* ref. 9). In our hands, a 1:500 RNase dose was used to make both the iCLIP and iCLAP libraries.
10. For better reproducibility of the iCLIP libraries, be consistent with the timing of the RNase treatment.
11. For the initial optimization of IPs (including control IPs) use a smaller amount of protein G dynabeads (~50 μl) per IP assay.
12. For every new antibody the initial experiment requires testing the optimal antibody concentration that will be coupled to dynabeads.
13. At this point separate the dynabeads into 100 and 900 μl fractions. The 100 μl fraction will be used for 5′ RNA radiolabeling while the lack of a 3′ RNA linker will prevent reverse transcription. The 900 μl fraction will be used for 3′ RNA linker attachment.
14. Before the 3′-linker is ligated to RNA, it must be dephosphorylated, since the T4 RNA ligase cannot add ends to the cyclic 2–3′ phosphate groups.
15. To increase the specificity of the immunoprecipitation you can increase the time or the number of high salt washes.

Fig. 3 (continued) linked RNA–protein pull-down in the presence of high RNase I treatment (lane 4) serve as additional negative controls. (**b**) Optimization of the MRP1 iCLIP libraries. The first panel shows the final MRP1 iCLIP library PCR carried out using 25 cycles that resulted in a smear. Decreasing the number of PCR cycles to 20 resulted in sharper bands

16. Protein spin columns can be used for easy washing and resuspension of the IgG beads.
17. It is important to use homemade GST-TEV protease since most commercially available TEV proteases contain a His tag. This will create a problem in the second step of His-tag purification of the protein complex.
18. The time for TEV protease cleavage might vary depending upon the batch of TEV protease or the RBP of interest and therefore requires initial optimization.
19. One can also use nickel- or copper-based Sepharose beads. However, it is easier to work with dynabeads, since some downstream steps require resuspension of beads in smaller volumes. For example, 3′ RNA dephosphorylation and linker ligation uses 10 μl of total master mix.
20. The cross-linked RNA adds additional weight to the RBP. Therefore, if there is no other contaminating RBP in the vicinity (as already determined from the high RNase treated pull-down experiment), cut out the nitrocellulose membrane ~20–60 kDa above the expected molecular weight of the actual RBP.
21. In the original iCLIP protocol, the authors tested all the available RTCLIP primers. In their examination RTCLIP primers 1, 2, 6, 9, 10, and 13–16 were more efficient. As every RTCLIP primer contains a unique barcode, it allows the pooling of various iCLIP libraries to a single lane by using different RTCLIP primers. Every time prepare a fresh dilution of RCLIP primers (0.1 μM). Names and sequences of all primers used in the iCLIP/iCLAP protocol are provided in Fig. 4.
22. Remember to include the negative control for the reverse transcriptase reaction.
23. Use a fresh scalpel for samples with different RTCLIP primer barcodes. Cut the higher band first and then move downward to avoid cross contamination of primer dimers (runs around ~51 nt). It is crucial to avoid primer dimers in the library preparation, as they considerably decrease its depth.
24. Circularization and linearization make the identification of UV-cross-linked peptide position easy to decipher by bioinformatics, since the first nucleotide after the removal of the adapter from iCLIP sequence becomes the UV-cross-linked site.
25. The aim is to pool different size fractions in a ratio that will allow similar sequencing depths for each fraction (*see* Fig. 3b).

Primer's name	Sequence Information
RT1	/5Phos/NNAACCNNNAGATCGGA AGAGCGTCGTGgatcCTGAACCGC
RT2	5Phos/NNACAANNNA GATCGGAAGAGCGTCGTGgatcCT GAACCGC
RT3	/5Phos/NNATTGNNNAGATCGGA AGAGCGTCGTGgatcCTGAACCGC
RT4	/5Phos/NNAGGTNNNAGATCG GAAGAGCGTCGTGgatcCTGAA CCGC
RT6	/5Phos/NNCCGGNNNAGATC GGAAGAGCGTCGTGgatcCTGAA CCGC
RT7	/5Phos/NNCTAANNNAGATCGGA AGAGCGTCGTGgatcCTGAACCGC
RT8	/5Phos/NNCATTNNNAGATCGGA AGAGCGTCGTGgatcCTGAACCGC
RT9	/5Phos/NNGCCANNNAGATCGG AAGAGCGTCGTGgatcCTGAACCGC
RT11	/5Phos/NNGGTTNNNAGATCGGAAGAGCGTCGTGgatcCTGAACCGC
RT12	/5Phos/NNGTGGNNNAGA TCGGAAGAGCGTCGTGgatcCT GAACCGC
RT13	/5Phos/NNTCCGNNNAGATCGGAA GAGCGTCGTGgatcCTGAACCGC
RT14	/5Phos/NNTGCCNNNAG ATCGGAAGAGCGTCGTGgatc CTGAACCGC
RT15	/5Phos/NNTATTNNNAGATCGGAA GAGCGTCGTGgatcCTGAACCGC
RT16	/5Phos/NNTTAANNNAGATCGGAAG AGCGTCGTGgatc CTGAACCGC
Cut oligo	GTTCAGGATCCACGACGCTCTTCaaaa
L3 linker	rAppAGATCGGAAGAGCGGTTCAG/ddC/
P5	AATGATACGGCGACCACCGAGA TCTAC ACTCTTTCCCTACACGA CGCTCTTCCGATCT
P3	CAAGCAGAAGACGGCATACGA GATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT

Fig. 4 Primers required for preparation of the library

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