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Chapter 6

Mapping Exosome–Substrate Interactions In Vivo by UV Cross-Linking

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Abstract

The RNA exosome complex functions in both the accurate processing and rapid degradation of many classes of RNA in eukaryotes and Archaea. Functional and structural analyses indicate that RNA can either be threaded through the central channel of the exosome or more directly access the active sites of the ribonucleases Rrp44 and Rrp6, but in most cases, it remains unclear how many substrates follow each pathway in vivo. Here we describe the method for using an UV cross-linking technique termed CRAC to generate stringent, transcriptome-wide mapping of exosome–substrate interaction sites in vivo and at base-pair resolution.

Key words Exosome, RNA processing, RNA degradation, Protein–RNA interaction, RNA-binding sites, UV cross-linking, Yeast

1 Introduction

We present a protocol for the identification of RNA interaction sites for the exosome, using UV *cr*oss-linking and *a*nalysis of *c*DNA (CRAC) [1, 2]. A number of related protocols for the identification of sites of RNA–protein interaction have been reported, including HITS-CLIP, CLIP-Seq, iCLIP, eCLIP, and others [3–6]. These all exploit protein immunoprecipitation to isolate protein–RNA complexes. CRAC is distinguished by the inclusion of tandem affinity purification and denaturing purification, allowing greater stringency in the recovery of authentic RNA–protein interaction sites.

To allow CRAC analyses, strains are created that express a "bait" protein with a tripartite tag. This generally consists of His6, followed by a TEV-protease cleavage site, then two copies of the z-domain from Protein A (HTP). The tag is inserted at the C terminus of the endogenous gene within the chromosome. The fusion construct is the only version of the protein expressed and this is under the control of the endogenous promoter. Several alternative tags have been successfully used, including a version with

N-terminal fusion to a tag consisting of $3\times$ FLAG-PreSission protease (PP) cleavage site-His6 (FPH) [7]. This is a smaller construct and is suitable for use on proteins with structures that are incompatible with C-terminal tagging. An additional variant is the insertion of a PP site into a protein that is also HTP tagged. This allows the separation of different domains of multidomain proteins. Importantly, the intact protein is cross-linked in the living cell, with domain separation in vitro. This has been successfully applied to the exosome subunit Rrp44/Dis3 to specifically identify binding sites for the PIN endonuclease domain [8].

Briefly, during standard CRAC analyses, covalently linked protein–exosome complexes are generated in vivo by irradiation with UV-C (254 nm). This generates RNA radicals that rapidly react with proteins in direct contact with the affected nucleotide (zero length cross-linking). The cells are then lysed and complexes with the bait protein are purified using an IgG column. Protein–RNA complexes are specifically eluted by TEV cleavage of the fusion protein and cross-linked RNAs trimmed using RNase A/T1, leaving a protected "footprint" of the protein binding site on the RNA. Trimmed complexes are denatured using 6 M Guanidinium, immobilized on Ni-NTA affinity resin and washed under denaturing conditions to dissociate copurifying proteins and complexes. The subsequent enzymatic steps are all performed on-column, during which RNA 3′ and 5′ ends are prepared, labeled with ^{32}P (to allow RNA–protein complexes to be followed during gel separation) and linkers ligated. Note, however, that alternatives to using ^{32}P labeling have been reported (e.g., [6]). The linker-ligated, RNA–protein complexes are eluted from the Ni-NTA resin and size selected on a denaturing SDS-PAGE gel. Following elution, the bound RNA is released by degradation of the bait protein using treatment with Proteinase K. The recovered RNA fragments are identified by reverse transcription, PCR amplification and sequencing using an Illumina platform.

Relative to CLIP-related protocols, CRAC offers the advantages of stringent purification, that substantially reduces background, and on-bead linker ligation that simplifies separation of reaction constituents during successive enzymatic steps. It also avoids the necessity to generate high-affinity antibodies needed for immunoprecipitation. Potential disadvantages are that, despite their ubiquitous use in yeast studies, tagged constructs may not be fully functional. This can be partially mitigated by confirming the ability of the tagged protein to support normal cell growth and/or RNA processing, or by comparing the behavior of N- and C-terminal tagged constructs. Additionally, because linkers are ligated to the protein–RNA complex, a possible disadvantage is that UV-cross-linking of the RNA at, or near, the 5′ or 3′ end it may sterically hinder on-column (de)phosphorylation and/or linker ligation. With these caveats, CRAC has been successfully

applied to >50 proteins in budding yeast, and in other systems ranging from pathogenic bacteria to viral infected mouse cells [7, 9].

2   Materials

All steps should be performed wearing disposable gloves and materials should be free of DNase and RNase. Prior to each CRAC experiment, pipettes should be cleaned with DNAZap (Thermo-Fisher; AM9890) to avoid DNA contamination at the PCR step, followed by RNaseZAP (ThermoFisher; AM9890) treatment, and rinsed with deionized water. All the buffers should be prepared with deionized water and free of RNases; however, DEPC treatment is not normally essential. To minimize buffer contamination, adjust the pH by taking small aliquots for measurements. Filter-sterilize stock solutions following preparation, and store at 4 °C. Where required, add β-mercaptoethanol and protease inhibitors to the buffers shortly before use. Wash buffers should be prepared immediately before starting the CRAC experiment.

2.1   Yeast Strains and Culture Media

2.1.1   Yeast Strains

Purification of the RNA–protein complex requires that the protein of interest is tagged, generally with the HTP (His × 6—TEV protease cleavage site—Protein A × 2) tandem affinity tag [1, 2]. In order to study RNA targets of the exosome, strains were prepared carrying tagged, intact Rrp44 and versions that lacked exonuclease or endonuclease activity, expressed from the chromosomal *RRP44* locus or from a single copy plasmid in *rrp44Δ* strains. Both were studied by CRAC to confirm that recovered RNAs are similar [10]. Then, strains expressing mutant and wild-type versions of Rrp44 from a single copy plasmid were used for CRAC.

We also tagged genomic copies of the nuclear exosome exonuclease Rrp6, the exosome core subunits Csl4 (exosome cap) and Rrp41 (exosome channel), and both wild-type and mutated components of the TRAMP complex (exosome cofactors) Mtr4, Mtr4-arch, Air1, Air2, Trf4 and Trf5. The untransformed, parental yeast strain (BY4741) was used as a negative control throughout the analyses.

2.1.2   Growth Media

Tryptophan absorbs 254 nm light, potentially interfering with cross-linking, and should be omitted from growth media. We use Yeast Nitrogen Base (YNB, Formedium) supplemented with 2% glucose and amino acids without tryptophan, unless other amino acids need to be omitted for plasmid maintenance.

| **2.2 Buffers and Solutions** | To avoid potential contamination, check pH of buffers by pipetting a small volume onto pH paper. |

1. Phosphate-buffered saline (PBS).

2. TN150-Lysis buffer: 50 mM Tris–HCl pH 7.8, 150 mM sodium chloride, 0.1% Nonidet P-40 substitute (Roche), 5 mM β-mercaptoethanol, one tablet of EDTA-free cOmplete protease inhibitor cocktail (Roche, 11697498001) per 50 ml solution.

3. TN1000 buffer: 50 mM Tris–HCl pH 7.8, 1 M sodium chloride, 0.1% Nonidet P-40 substitute (Roche), 5 mM β-mercaptoethanol.

4. TN150 buffer: 50 mM Tris–HCl pH 7.8, 150 mM sodium chloride, 0.1% Nonidet P-40 substitute (Roche), 5 mM β-mercaptoethanol.

5. Wash buffer I: 6 M guanidine hydrochloride, 50 mM Tris–HCl pH 7.8, 300 mM sodium chloride, 10 mM imidazole pH 8.0, 0.1% Nonidet P-40 substitute (Roche), and 5 mM β-mercaptoethanol.

6. Wash buffer II: 50 mM Tris–HCl pH 7.8, 50 mM sodium chloride, 10 mM imidazole pH 8.0, 0.1% Nonidet P-40 substitute (Roche), and 5 mM β-mercaptoethanol.

7. 1× PNK buffer: 50 mM Tris–HCl pH 7.8, 10 mM magnesium chloride, 0.1% Nonidet P-40 substitute (Roche), 5 mM β-mercaptoethanol.

8. 5× PNK buffer: 250 mM Tris–HCl pH 7.8, 50 mM magnesium chloride, 25 mM β-mercaptoethanol.

9. Elution buffer: 50 mM Tris–HCl pH 7.8, 50 mM sodium chloride, 150 mM imidazole pH 8.0, 0.1% Nonidet P-40 substitute (Roche), 5 mM β-mercaptoethanol.

10. Proteinase K buffer: 50 mM Tris–HCl pH 7.8, 50 mM sodium chloride, 0.1% Nonidet P-40 substitute (Roche), and 5 mM β-mercaptoethanol, 1% sodium dodecyl sulfate (v/v), 5 mM EDTA.

11. 1 M Tris–HCl pH 7.8.

12. 0.5 M EDTA [Ethylenediaminetetraacetic acid disodium salt dihydrate] pH 8.0.

13. Guanidine HCl [Guanidinium].

14. 5 M sodium chloride.

15. 2.5 mM imidazole pH 8.0.

16. Trichloroacetic acid (TCA).

17. Acetone.

18. Methanol.

19. Proteinase K solution (20 mg/ml).

20. 3 M sodium acetate pH 5.2.

21. 25:24:1 phenol–chloroform–isoamyl alcohol mixture.

22. 100% and 70% ethanol (stored at −20 °C).

23. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA.

24. Deionized water.

2.3 Enzymes and Enzymatic Reaction Components

1. TEV protease (do not use His-tagged TEV as this will be recovered on the Ni column).

2. Thermosensitive alkaline phosphatase (TSAP) (Promega, M9910).

3. RNasin RNase inhibitor (Promega, N2511, red cap).

4. T4 RNA ligase 1 (New England Biolabs, M0204S).

5. [γ^{32}P] ATP (6000 Ci/mmol, Hartmann Analytic)

6. 10 mM deoxyribonucleotides (10 mM each) (Sigma-Aldrich, D7295).

7. Superscript III and accompanying 5× first strand buffer (Invitrogen, 18080044).

8. 100 mM DTT (Invitrogen, accompanies 18080044)

9. RNase H (New England Biolabs, M0297S).

10. LA Taq polymerase (TaKaRa, RR002M).

11. 10× LA Taq PCR Buffer (TaKaRa, accompanies RR002M).

12. RNace-IT (Agilent) RNase A+T1, working stock prepared by diluting 1:100 in water, store long term at −20 °C.

13. ATP, 100 mM and 10 mM solutions in water, aliquot and store at −20 °C, avoid repeated freezing and thawing.

14. T4 PNK, T4 Polynucleotide Kinase (New England BioLabs, M0201L).

15. Proteinase K (Roche Applied Science), prepare 20 mg/ml stock in deionized water, aliquot and store at −20 °C.

2.4 Oligonucleotides

All oligonucleotides were supplied by Integrated DNA Technologies (IDT) and are listed in Table 1. The forward and reverse PCR primers introduce sequences that allow binding of the PCR product to an Illumina flow cell. Illumina compatible adapters, RT and PCR primers: miRCat-33 Conversion Oligos Pack (miRCat-33 adapter and miRCat-33 RT primer; IDT), other oligonucleotides synthesized by custom order.

After dissolving, prepare aliquots of adapters and store at −80 °C.

Table 1
Oligonucleotides used in CRAC experiments

Illumina barcoded 5′ adapter	L5Aa	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr UrArArGrC-OH**
	L5Ab	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr ArUrUrArGrC-OH**
	L5Ac	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr GrCrGrCrArGrC-OH**
	L5Ad	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr CrGrCrUrUrArGrC-OH**
	L5Ba	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr ArGrArGrC-OH**
	L5Bb	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr GrUrGrArGrC-OH**
	L5Bc	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr CrArCrUrArGrC-OH**
	L5 Bd	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr UrCrUrCrUrArGrC-OH**
	L5Ca	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr CrUrArGrC-OH**
	L5Cb	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr UrGrGrArGrC-OH**
	L5Cc	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCrUr**NrNrNr ArCrUrCrArGrC-OH**
	L5Cd	invddT-ACACrGrArCrGrGrCrUrCrUrUrCrCrGrArUrCr**rNrNrNr GrArCrUrUrArGrC-OH**
Illumina 3′ adapter	miRCAT 33	AppTGGAATTCTCGGGTGCCAAG/ddC/'
RT primer	miRCat RT	CCTTGGCACCCGAGAATT
PCR primers	P5_Fwd	AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGACGCTCTTCCGATCT
	PE- miRCat_Rev.	CAAGCAGAAGACGGCATAC GACCTTGGCACCCGAGAATTCC

2.5 Laboratory Equipment

1. Incubator with orbital shaker.

2. UV cross-linker (Megatron, UVO3). Megatron parts were purchased from UVO3 (http://www.uvo3.co.uk).

3. Refrigerated centrifuge for 1 l bottles.

4. Refrigerated centrifuge for 50 ml and 15 ml centrifuge tubes.

5. Temperature controlled dry block (with range 16–65 °C) with shaking (preferentially two blocks).

6. Refrigerated microcentrifuge.

7. SDS-PAGE tank XCell SureLock Mini-Cell for NuPAGE gels.

8. Mini Trans Blot Electrophoretic Transfer Cell (wet-transfer apparatus for Western blotting) (Bio-Rad).

9. Phosphorimaging cassette.

10. Film developer.

11. Bunsen burner.

12. Thermocycler for cDNA synthesis.

13. Magnetic stirrer/hot plate.

14. Apparatus for agarose gel electrophoresis.

15. Gel scanner attached to printer, able to print gel scan in its original size.

16. Qubit 3.0 Fluorometer (Thermo Scientific).

17. Vortexer.

18. Geiger counter.

19. Laboratory room with authorization to work with radioactivity.

2.6 Other Consumables and Labware

1. Culture materials: 50 ml and 500 ml flasks for preculture, 4 l flasks for culture.

2. Filter units for buffer sterilization with pore size 0.2 μm.

3. RNase-free filter pipette tips.

4. SD medium: CSM −Trp and CSM −Trp −Leu (Formedium) for strains requiring plasmid maintenance with Leucine auxotrophic marker with 2% glucose and yeast nitrogen base (3 l of medium per sample).

5. 0.1 mm Zirconia beads.

6. IgG Sepharose® 6 Fast Flow (GE Healthcare, 17-0969-01).

7. Spin columns (Pierce, Snap Cap).

8. Ni-NTA resins (Qiagen, 30210).

9. 1.5 ml microcentrifuge tubes.

10. GlycoBlue (Ambion, AM9515) or glycogen for RNA/Protein precipitation.

11. NuPAGE bis-Tris 4–12% precast gradient gels (Invitrogen, NP0322BOX). This system is essential due to its high pH stability through the run.

12. NuPAGE LDS Sample Buffer, 4× (Life Technologies).

13. MOPS running buffer (Invitrogen, NP0001).

14. NuPAGE transfer buffer (Invitrogen, NP0006).

15. Nitrocellulose membranes (Thermo Scientific or GE Healthcare).

16. Phosphorescent rulers for autoradiography.

17. Kodak BioMax MS Autoradiography Film.

18. DNA Gel extraction kit with low elution volumes (e.g., MinElute Gel extraction kit (Qiagen)).

19. Transparency film.

20. MetaPhor high resolution agarose (Lonza, 50181).

21. SYBR Safe (Life Technologies, S33102).

22. 50 bp DNA ladder (e.g., GeneRuler 50) and loading dye (e.g., GeneRuler DNA Ladder Mix by Thermo Scientific, SM0331)

23. Prestained protein standard SeeBlue Plus2 (Life Technologies, LC5925).

24. Scalpels.

25. Qubit dsDNA HS Assay Kit (Life Technologies, Q32851).

3   Methods

Appropriate negative controls and experimental replicates are required to determine the background signal and true positive binding sites. We routinely use the (untagged) yeast parental strain as a negative control, performing a minimum of two biological and technical replicates for each sample. It is commonly observed that technical replicates (even samples from the same culture) processed in two independent CRAC experiments show more differences than two biological replicates (independent cultures) processed together.

3.1   Cell Culture and UV Cross-Linking

3.1.1   Growth and Cross-Linking of RNA–Exosome Subunit-His-TEV-ProteinA (HTP) Complexes

1. Streak out HTP-tagged and negative control strains from glycerol stocks onto YPD plates (or SD medium—leucine when required for plasmid maintenance) and incubate at 30 °C for 48–76 h.

2. Inoculate single colonies into 5 ml of SD medium (YNB−amino acids, CSM−TRP, and 2% glucose) and incubate at 30 °C with shaking at 200 rpm for 6 h, then use to inoculate 100 ml overnight cultures.

3. Inoculate 2.75 l of prewarmed minimal media with the quantity of overnight culture required to obtain an OD_{600} around 0.05, and grow until the OD_{600} measurement reaches 0.5.

4. Clean the UV cross-linker with deionized water before use, and prewarm the lamp.

5. Irradiate cultures in the UV cross-linker at room temperature for 100 s (254 nm, equivalent to an average dose of $\sim$1.6 mJ cm^{-2}).

6. Pour the cultures into prechilled 1 l centrifuge bottles on ice. Harvest by centrifugation; $2700 \times g$ for 15 min at 4 °C.

7. Resuspend pellet in 30 ml of ice-cold phosphate-buffered saline (PBS) and transfer to a 50 ml Falcon tube. Centrifuge for 5 min

at 3000 × g at 4 °C. We usually keep the three pellets in individual tubes and use one per CRAC experiment.

8. Discard supernatant and store pellets at −80 °C until required.

3.2 Cell Lysis, Two-Step Purification, and RNase Digestion

All steps must be carried out on ice, unless stated otherwise. For troubleshooting, it is a good idea to monitor the course of the experiment by retaining samples at points during the CRAC protocol. This allows potential problems with Protein–RNA purification steps to be identified. Three aliquots per sample are taken during the purification (Subheading 3.2.2 "Crude Lysate" and "IgG supernatant," Subheading 3.2.3 "TEV Eluate"). These can be analyzed by Western blot.

3.2.1 Cell Lysis

1. Add one volume (1 ml) of TN150 lysis buffer (+ protease inhibitors) to cell pellets and thaw rapidly. 1 l of cell culture yields pellets of approximately 1 ml, but lysis volumes should be increased if larger cultures were used.

2. Add 2.5 volumes of Zirconia beads to cell pellet (~ 2.5 ml, measure in 15 ml Falcon tubes). Vortex the cells 1 min, then put them on ice for 1 min between each vortexing step. Repeat five times. Using a powerful vortex increases the efficiency of the lysis.

3. Add three volumes of TN150 (+ protease inhibitors) (~3 ml), shake vigorously, and centrifuge the suspension in the falcon tube (20 min at 4000 × g, 4 °C).

4. Transfer the supernatant (~ 4.5 ml) to three 1.5 ml tubes and spin lysate for 20 min at 16,000 × g in a microcentrifuge at 4 °C. Keep tubes on ice throughout the process.

5. Keep supernatants on ice, ready to be mixed with IgG sepharose beads in Subheading 3.2.2, **step 4** below.

3.2.2 Protein A:IgG Purification

1. Resuspend IgG Sepharose beads by gently inverting the bottle. Pipette out 167 μl of stock per sample into a 15 ml tube. The bead:buffer ratio is approximately 75:25 so this equates to 125 μl of beads. Wash the beads twice with 5 ml of TN150 lysis buffer + protease inhibitors. Collect beads between washes by pulsing in the centrifuge to 1000 rpm (210 × g). Use wide orifice pipette tips or use a sterile scalpel to cut the end off of a regular pipette tip for more efficient pipetting of the beads.

2. Remove the supernatant after the second wash and add 125 μl per sample of TN150 + protease inhibitors. Gently resuspend the beads, and aliquot 250 μl of this mix per sample into 15 ml tubes.

3. Take 5 μl aliquots of the cleared lysate ("crude lysate") for troubleshooting the purification, and store at −20 °C.

4. Mix remaining lysates with IgG Sepharose beads and rotate for a minimum of 2 h at 4 °C. This step can be extended to overnight.

5. Collect the beads by pulsing to 1000 rpm at 4 °C and remove most of the supernatant.

6. Take a 5 µl aliquot of supernatant for troubleshooting ("IgG supernatant") and store at −20 °C.

7. Wash beads twice with 10 ml of TN1000 and twice with 10 ml of TN150. Buffers used in this and all subsequent steps should not contain protease inhibitors. For each wash, gently agitate at 4 °C for 5 min.

8. Gently resuspend gently the beads in 600 µl of TN150 and transfer to a 1.5 ml tube.

3.2.3 TEV Cleavage

1. Add 20–30 units of TEV protease to the beads and mix by inverting tube.

2. Incubate at 18 °C for 2 h with shaking (make sure beads remain in suspension).

3. Pass the mixture through a microcentrifuge column (SnapCap) to remove the beads. Spin column (1000 rpm) to collect all the eluate in a 1.5 ml tube. Do not put eluate on ice.

4. Take a 25 µl sample of eluate ("TEV eluate") and store at −20 °C.

3.2.4 RNase Digestion and Binding to Ni-NTA Resin

The concentration of RNaceIT used to footprint (trim) RNAs on protein of interest is determined empirically. Ideally, the reads will be long enough to map uniquely (~17 nt) but short enough to give good resolution of the protein-binding site. We aim to generate an average RNA length of ~30 nt. Commercially available RNase stock is highly concentrated, so to minimize discrepancies between experiments it is practical to prepare a working stock of RNases (we use 1:100 dilution in water), store it at 4 °C and use for all subsequent experiments.

1. Prepare 1.5 ml microcentrifuge tubes containing 0.4 g of guanidine HCl.

2. Prepare 50 µl of Ni-NTA resin by pipetting 100 µl of 50% slurry into tubes and wash them twice with 1 ml of Wash Buffer I. Leave the resin in about 100 µl of Wash Buffer I until use.

3. Add 1 µl of a 1:100 dilution of RNace IT (0.15 units, Agilent) to 550 µl of the TEV eluate. Incubate for 5 min at 37 °C.

4. Immediately transfer the RNase treated eluate into the prepared microcentrifuge tubes containing guanidine hydrochloride. The final volume will be around 700 µl.
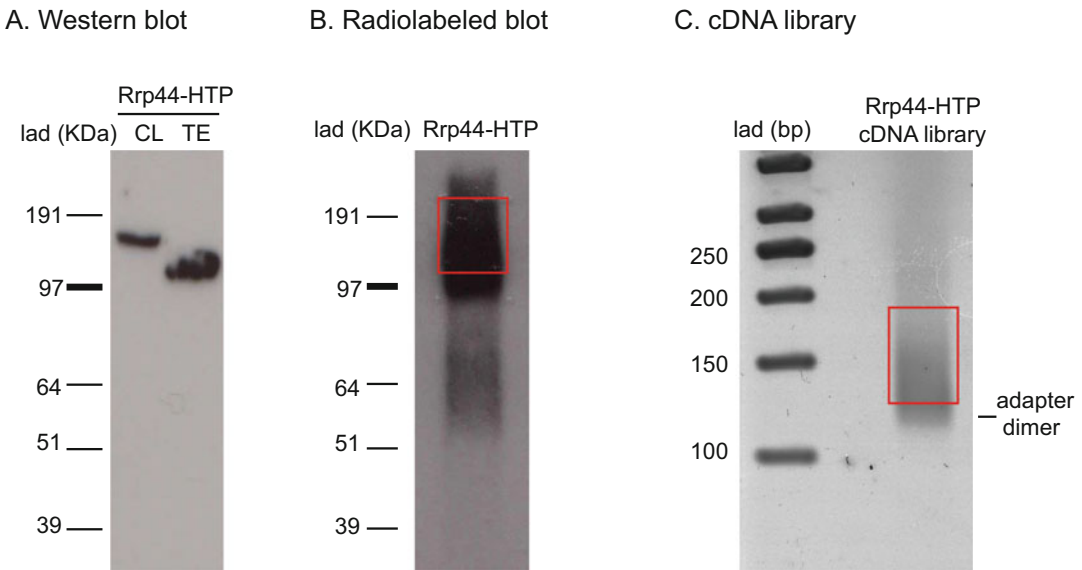
A. Western blot   B. Radiolabeled blot   C. cDNA library

Fig. 1 Key steps of Rrp44-HTP CRAC protocol. (**a**) Western blot to test purification of Rrp44 after lysis (Crude Lysate or CL sample) and after elution from IgG beads and TEV cleavage (TEV eluate or TE sample). Difference in size is due to cleavage of part of the tag (Protein A × 2) by TEV protease. (**b**) Autoradiogram of labeled RNAs cross-linked to HTP-Rrp44. The part of the membrane cut and subjected to proteinase K treatment is indicated in red. (**c**) cDNA library (product of PCR amplification) resolved on Metaphor agarose gel. The region of the gel that was cut for size selection and gel purification of the library is indicated in red

5. Add 27 μl of 5 M NaCl (300 mM final concentration) and 3 μl of 2.5 M pH 8.0 imidazole (10 mM final concentration) to the eluate.

6. Remove the remaining supernatant from the washed Ni-NTA resin. Add the eluate to the resin.

7. Incubate with gentle agitation at 4 °C overnight.

8. Pulse the microcentrifuge tubes to collect the resin. Remove the supernatant and wash three times with 400 μl of Wash Buffer I and three times with 400 μl of 1× PNK buffer.

9. Transfer the resin to a microcentrifuge column (SnapCap).

10. Western blot with "Crude Lysate," "IgG supernatant," and "Tev Eluate" controls can be carried out in parallel of the steps below (Fig. 1a). If needed, additional controls can be prepared by taking aliquots of the experiments in other steps, such as pipetting out 30 μl of IgG beads before addition of TEV protease and 30 μl after TEV treatment to control binding and cleavage efficiency.

3.3 Linker Ligation at both Ends of RNAs on Beads

Enzymatic reactions are performed on beads (Ni-NTA resin) contained in Snap cap columns. A metal rack for 1.5 ml microcentrifuge tubes greatly simplifies working with these columns by helping them being vertical and cold when placed in the ice bucket. To prevent contamination caused by buffer dripping from the column, it is very important to first open the column lid, then open the press-on bottom stopper, before transferring the column between tubes. It is also essential to close the bottom of columns before closing the cap.

All washes are performed under gravity flow. However, it happens that some batches of columns do not drip, or drip really slowly; in that case centrifugation might be necessary.

Guanidine contained in Wash Buffer I inhibits enzymatic reactions and must be removed completely before each enzymatic step. For efficient removal of guanidine traces, Wash Buffer 1 should be pipetted directly onto the beads at the bottom of the column. Then, $1\times$ PNK buffer should be pipetted so that it rinses the side of the columns.

3.3.1 Dephosphorylation of RNA 3′ P Ends Using Alkaline Phosphatase (TSAP)

TSAP catalyzes removal of 5′ and 3′ phosphate groups from DNA and RNA; it is effective on 3′ overhangs, 5′ overhangs and blunt ends and leaves 5′ OH and 3′ OH ends. Treating the RNAs with alkaline phosphatase will remove the 3′ phosphates left behind by the RNase cleavage of the RNA.

1. Spin out the residual $1\times$ PNK buffer and close the column with the supplied press-on bottom stopper. To each sample, add 80 μl of TSAP master mix that contains: 16 μl of $5\times$ PNK buffer, 8 μl of TSAP, 2 μl of RNasin, and 54 μl of Milli-Q water. Mix by stirring with a pipette tip then flicking column gently.

2. Incubate at 37 °C for 30 min.

3. Wash the resin once with 400 μl of Wash Buffer I and three times with 400 μl of $1\times$ PNK buffer.

3.3.2 On Bead Ligation of 3′ miRCat-33 Linker

The 3′-linker is a DNA oligonucleotide that has a blocked 3′ end to prevent self-ligation and a 5′-end that is preactivated by adenylation (AppN...). T4 RNA ligase usually activates its substrate by preadenylation using ATP. Employing a preadenylated linker allows the reactions to be performed in the absence of ATP. This decreases the risk of circularizing any remaining 5′-phosphorylated RNA; a side reaction that would otherwise be expected. Moreover, addition of ATP in the mix could inhibit the reaction, as the active site of T4 RNA ligase would get adenylated and could not transfer the adenosine to any substrate as the linker is already adenylated.

1. Spin out the residual volume of $1\times$ PNK buffer. Close the bottom of the column with the press-on stopper. To each sample, add 76 μl of miRCat master mix containing: 16 μl of

5× PNK buffer, 8 µl of 10 µM miRCat-33, 2 µl of RNasin, and 50 µl of Milli-Q water.

2. To each sample add 4 µl of T4 RNA ligase I.

3. Incubate at 25 °C for 4 h.

4. Wash the resin once with 400 µl of Wash Buffer I and three times with 400 µl of 1× PNK buffer.

3.3.3 Phosphorylating the 5′ Ends of Cross-Linked RNA

The labeling reaction is performed first with radioactive ATP only to obtain a reasonable radioactive signal from the RNA in the sample. Subsequently, nonlabeled ATP is added to the reaction to allow efficient phosphorylation of all RNA 5′ ends in the sample, required for 5′ end adapter ligation.

1. Spin out the residual volume of 1× PNK buffer. Cap the bottom of the column. To the resin, add 80 µl of PNK master mix containing: 16 µl of 5× PNK buffer, 4 µl of T4 polynucleotide kinase, 56 µl of Milli-Q water, and 4 µl of ^{32}P-ATP (10 µCi/µl). Incubate at 37 °C for 40 min.

2. Add 1 µl of 100 mM ATP and continue the incubation for 20 min.

3. Wash the resin four times with 400 µl of Wash Buffer I and three times with 1× PNK buffer. Additional washes can be done to remove most free radioactive ATP and decrease the chance of radioactive contamination at later stages. Perform the washes until the radioactivity of the flow through measured with a manual Geiger counter falls to approximately 10–15 cps.

3.3.4 On-Column Ligation of the 5′ Adapters

These linkers have blocked 5′ end to prevent self-concatenation. Moreover, they contain barcodes allowing distinction of samples in case of multiplexing and random nucleotides to distinguish molecules with same 5′- and 3′-end (allowing removal of PCR duplicates). It is crucial to use different barcodes for each sample.

1. Spin out the residual volume of 1× PNK buffer. Add 75 µl of 5′ linker master mix containing: 16 µl of 5× PNK, 8 µl of 10 mM ATP, 2 µl of RNasin, 49 µl of Milli-Q water.

2. To each sample add 1 µl of barcoded 5′ linker and 4 µl of T4 RNA ligase I.

3. Incubate overnight at 16 °C.

4. Wash the resin three times with Wash Buffer II.

3.4 Elution, SDS-PAGE, and RNA Purification

3.4.1 Elution and Precipitation of Exosome Subunit–RNA Complexes

1. Spin out the void volume. Close the bottom of the column with the press-on stopper and add 200 µl of Elution buffer.

2. Incubate the resin on ice for 5 min. Alternatively, to increase efficiency, incubation can be performed at 16 °C with shaking.

3. Collect the flow-through in a 1.5 ml microcentrifuge tube. Reclose the bottom of the column and repeat the elution with another 200 µl of Elution buffer. Use the Geiger counter to ensure that the elution flow-through is radioactive.

4. Collect the residual Elution buffer on the column by briefly spinning the column.

5. Pool the eluates into a single microcentrifuge tube. Add 40 µg of GlycoBlue coprecipitant and 100 µl of trichloroacetic acid. Vortex and incubate on ice for 1 h. Alternatively, you can elute in 2×100 µl, add 40 µg of GlycoBlue coprecipitant and 1 ml of acetone to precipitate overnight at -20 °C (and skip **step 7**).

6. Centrifuge at top speed for 30 min at 4 °C. Remove the supernatant (use the Geiger counter to ensure that the pellet has not been dislodged if the blue pellet is not visible).

7. Add 800 µl of ice-cold acetone to the pellet and centrifuge for 20 min at 4 °C. Pellets should be small and clear. If it is big and white, add additional acetone wash step and pipette up and down until the pellet has dissolved completely. Longer incubation with acetone on ice can also be considered.

8. Remove the supernatant and air-dry the pellet at room temperature. Pellets can be hard to resuspend; do not allow them to overdry as this can lead to loss of material during the resuspension step.

3.4.2 PAGE Separation and Transfer

1. Resuspend the pellet in 30 µl of $1\times$ NuPAGE LDS sample buffer (dilute $4\times$ buffer in distilled water before use). Pipette along the wall of the tube very carefully and check with the Geiger counter to ensure that most of the material has been resuspended.

2. Heat the samples at 65 °C for 10 min.

3. Load the sample and SeeBlue2 ladder onto a NuPAGE 4–12% gradient gel. Run for 1 h at maximum 150 V or until the dye reaches the bottom of the gel.

4. Transfer the protein–RNA complex to nitrocellulose using a wet transfer western blotting system, at 100 V for 1.5 h in NuPage transfer buffer supplemented with 10% Methanol. To avoid overheating during transfer, the tank can be placed in ice.

5. Expose the membrane (wrapped in cling film or protected by a transparent plastic film) to a high-sensitivity X-ray film at -80 °C. If samples are highly radioactive, a 30–60 min

exposure time should be enough. Overnight exposure is often required for samples with weaker radioactive signal. Ensure that a chemiluminescent marker is included to realign membrane and film after developing.

6. Develop the X-ray film and align it to the membrane using the chemiluminescent rulers. Cut out the smear corresponding to the size of the protein–RNA complex for all the samples. Cut at the same place in the negative control lane. Use clean scalpel for each sample. The first incision can be made in the middle of the band corresponding to the protein of interest plus the smear above to get most cross-link species. Once membrane fragments have been excised, they can be stored overnight (or longer) at −20 °C or −80 °C. An example of a radiolabeled blot for Rrp44-HTP is shown in Fig. 1b.

3.4.3 Recovery of Trimmed, Adapter-Ligated RNA

1. To digest away proteins, incubate the membrane slices with 400 μl of Proteinase K buffer (50 mM Tris–HCl pH 7.8, 50 mM NaCl, 0.1% NP-40, 5 mM β-mercaptoethanol, 1% SDS, 5 mM EDTA). Add 100 μg of Proteinase K and incubate at 55 °C for 2 h with gentle mixing.

2. Add 50 μl of 3 M sodium acetate (pH 5.2) and 500 μl of phenol–chloroform–isoamyl alcohol (25:24:1). Vortex and centrifuge for 5 min at room temperature.

3. Transfer the aqueous phase to clean microcentrifuge tube and add 1 ml of ice cold absolute ethanol and 20 μg of GlycoBlue. Incubate at −80 °C for 30 min and centrifuge at $16,000 \times g$ and 4 °C for 30 min. Wash the pellet with 500 μl of ice cold 70% ethanol and centrifuge for 20 min. Aspirate the supernatant and air dry.

3.5 Generation of Library for Sequencing

3.5.1 Reverse Transcription of Purified RNA

To increase the efficiency of this step, prepare fresh dNTP dilution prior RT or aliquot and store at −20 °C to avoid multiple thawing.

1. Resuspend the RNA pellet in 11 μl of MilliQ water. Add 1 μl of RT primer [10 μM] and 1 μl of 10 mM dNTPs.

2. Heat the samples to 80 °C for 3 min, then chill on ice for 5 min. Collect the contents by brief centrifugation.

3. To each sample, add 4 μl of 5× First Strand buffer (Invitrogen), 1 μl of 100 mM DTT, and 1 μl of RNasin.

4. Incubate at 50 °C for 3 min and add 1 μl of SuperScript III (Invitrogen). This step will help dissociate any nonspecifically annealed primers from the RNA.

5. Incubate at 50 °C for 1 h.

6. Inactivate the Superscript III by incubating the samples at 65 °C for 15 min.

7. Add 2 μl of RNase H and incubate for 30 min at 37 °C.

3.5.2 PCR Amplification of cDNA Libraries

The number of cycles used to prepare cDNA libraries should be optimized for the template and limited to minimize artifacts due to overamplification, that is, the frequency of PCR duplicates. Generally, 21–22 cycles have been sufficient to produce complex libraries from cDNA generated from Exosome subunit-bound RNA, however we typically vary between 19 and 24 cycles and will increase number of independent PCR reactions (up to 5) for samples with low abundance of cDNA.

1. To 3 μl of cDNA template, add 47 μl of PCR master mix containing: 5 μl of 10× LA Taq buffer, 1 μl of 10 μM P5 Solexa primer, 1 μl of 10 μM pE_miRCat reverse primer, 5 μl of (fresh) 10 mM dNTPs, 0.5 μl of LA TaKaRa Taq polymerase, and 37.5 μl of nuclease-free water. We prepare three or more PCR reactions per sample to increase the complexity of our libraries.

2. The reaction is run with the following cycling conditions:

Temp	Time	Cycle
95 °C	2 min	
98 °C	20 s	21 cycles
52 °C	20 s	
68 °C	20 s	
72 °C	5 min	

3. Pool PCR reactions into a clean microcentrifuge tube and precipitate with 0.1 volume sodium acetate (pH 5.2) and 2.5 volumes of ice cold absolute ethanol. Incubate at −20 °C for 30 min (it is better to not precipitate longer to avoid recovering too much salt). Centrifuge at $16,000 \times g$ and 4 °C for 30 min. Remove the supernatant and air dry the pellet. Resuspend in 15 μl of MilliQ water. Alternatively, you can concentrate cDNA libraries using MinElute PCR purification kit as indicated in the manufacturer's instructions. Elute your samples with 20 μl water.

3.5.3 Size Selection of cDNA Libraries on Gel

At this stage, it is possible to adjust library size distribution and enrich the DNA library for cDNA of a certain length before sequencing. This size selection is dependent on the length of sequencing that will be used, the protein, and the biological questions CRAC is supposed to answer. If 50 bp sequencing length is planned, it is not useful to recover extra-long cDNAs; moreover longer sequences will decrease resolution of protein binding sites. On the other hand, for most proteins, it is preferable to avoid overpopulation of the library by short sequences (shorter than 20 nt), which are difficult to map confidently. In some case, these general guidelines have to be adjusted for biological relevance: for

instance, cDNA libraries from Rrp44-HTP are cut just above 130 nt to also recover short sequences enriched in cDNAs corresponding to RNAs bypassing the long exosome channel and directly accessing Rrp44.

1. Prepare a 3% Metaphor agarose gel using $1\times$ TBE buffer (with 1:1000 SYBR Safe) and store it at 4 °C for a minimum of 30 min. Preparing a Metaphor gel takes longer than preparing a standard agarose gel, and it is common for the agarose to form "lumps" which are hard to dissolve. One option is to let the Metaphor powder to soak for 30 min in $1\times$ TBE before agitating it on a magnetic stirrer hot plate. A second option is to microwave the mixture before agitating it on a magnetic stirrer hot plate. The gel can be prepared the day before and stored at 4 °C wrapped in cling film.

2. Add 5 µl of $6\times$ DNA gel loading dye to precipitated sample and load the entire volume onto the prepared 3% Metaphor agarose gel along with 50 bp DNA ladder.

3. Run the gel at 80 V for approximately 2 h or until the bromophenol blue dye front reaches 2 cm from the edge of the gel.

4. Image the gel. We use a Typhoon FLA9500 laser scanner (GE Life sciences) for increased sensitivity and print the gel images at 1:1 scale. A lower band around 120 nt corresponding to the amplified sequencing adapter dimers is sometimes visible and should be avoided when cutting. The cDNA libraries appear as a smear running above primer dimers that should be apparent in the negative control samples. The presence of a sharp band may indicate excessive RNA digestion. For other proteins, this can simply indicate the presence of a highly abundant binding target. However, this has not been observed with exosome components. Lack, or small amounts, of PCR products on the agarose gel (despite strong signal by autoradiography) suggests inefficient enzymatic reactions.

5. Place the gel on a transparent film and align it to the 1:1 scan of the gel. Excise the libraries using a sterile scalpel by cutting from the bottom of the smear to the predefined upper limit.

6. Transfer the gel slices to 2 ml microcentrifuge tubes. Rescan gel afterward to check the expected bits are cut out.

7. Add 1 ml of Buffer QG from the MinElute Gel Extraction purification kit (QIAgen) and incubate the gel slices at 42 °C for 15–20 min to dissolve the agarose.

8. Transfer the volume to a MinElute column fitted to collection tubes and spin for 1 min at 16,000 $\times$ g. Discard the flow-through. Repeat with the leftover of buffer/agarose to bind all the sample to the same column.

9. Add 750 μl Buffer QG and spin for 1 min at 16,000 × *g*. Discard the flowthrough.

10. Add 750 μl of Buffer PE (QIAgen) to the columns and incubate for 10 min at room temperature. Spin at 16,000 × *g* for 1 min and discard the flowthrough.

11. Dry the columns by spinning at 16,000 × *g* for 2 min at room temperature. Transfer the columns to clean 1.5 ml microcentrifuge tubes.

12. Add 20 μl MilliQ water on membrane and let stand for 2–5 min. Elute the purified cDNA by spinning at 16,000 × *g* for 1 min at room temperature.

13. Quantify the cDNA library using a Qubit high sensitivity DNA assay kit and fluorometer and store the libraries at −20 °C.

3.5.4   Sequencing

The samples can be submitted for single end sequencing on Illumina MiSeq, HiSeq, MiniSeq, or NextSeq platforms.

The read depth required for sufficient coverage of binding sites will depend on the number of RBP binding sites and complexity of the library generated (i.e., number of PCR duplicates). The exosome binds a huge diversity of targets. Since the highest proportion of the reads are aligned to ribosomal RNA, it is necessary to sequence deeply enough to detect less frequently bound targets. We generally aim to generate 17–35 nt trimmed RNA fragments that contain enough sequence information for a unique alignment, and that are short enough to ensure the protein interaction site is contained within the sequenced portion. We routinely use Illumina 50 bp single end sequencing, which is long enough to sequence into the 3′ adapter sequence.

3.6   Analysis of CRAC Datasets

Analysis of sequences obtained from exosome subunits CRAC experiments was done using custom scripts and software packages. The pyCRAC [11] software, a suite of python scripts which can be used to analyze sequencing data obtained from protein–RNA UV cross-linking protocols, includes most of the necessary tools. Here, we will describe the main steps of processing and the most commonly used modules of the pyCRAC software for our analysis.

3.6.1   Preprocessing Step: Demultiplexing, Quality Filtering, Trimming of Adapters

The 5′ adapters mentioned above contain barcodes allowing multiplexing of several samples in a sequencing lane. In addition to barcodes, 5′ adapters contain three random nucleotides allowing removal of PCR duplicates. This allows detection of reads with the same start and end positions that arise from PCR duplication of a single cDNA rather than independent linker ligation events.

For multiplexed samples, we first split the output file from sequencing by barcodes, using pyCRAC package.

```
$ pyBarcodeFilter.py -b barcodes.list -f multiplexed_input.
```

```
fastq
```

where barcodes.list is a tab-delimited text file containing the list of barcodes used in the experiment with corresponding names of samples, used in output files names. Here is an example of how the file should appear:

NNNTAAGC	Rrp44-HTP_L5Aa
NNNATTAGC	Rrp6-HTP_L5Ab
NNNGTGAGC	Rrp44-exo-HTP_L5Bb

The random nucleotides will be stripped in this step and will be placed into the header of each sequence of the ouput fastq files. Later steps can make use of this information in order to collapse PCR duplicates (*see* Subheading 3.6.2). It is important to note that the standard version of this script requires the adapters to be designed as shown in Table 1.

Sequencing data are then quality filtered and adapters trimmed using Flexbar (https://github.com/seqan/flexbar) [12] with parameters –at 1 –ao 4.

```
$ flexbar –r input.fastq –f solexa –as TGGAATTCTCGGGTGCCAAGG
–at 1 –ao 4 –u 3 –m 7 –n 16 –t flexbar.fastq
```

where input.fastq and flexbar.fastq are the input and output fastq files names respectively. When useful, for instance when proportion of 3′ oligoadenylated reads must be calculated (*see* Subheading 3.6.6), "-g" parameter can be added to tag reads with 3′ adapter. Then "grep" can be used to retain only these reads.

```
$ grep -A 3 --no-group-separator removal flexbar.fastq >
flexbar_adaptercontaining.fastq; done &
```

3.6.2 Collapsing

Then, sequences can be collapsed, thanks to the random nucleotides present in 5′ linker as mentioned in Subheading 3.6.1, using pyFastqDuplicateRemover.py script from pyCRAC software, so that reads having identical ends and identical random nucleotides in the 5′ barcode are counted as one.

```
$ nohup pyFastqDuplicateRemover.py -f flexbar.fastq -o flex-
bar_comp.fasta &
```

where flexbar_comp.fasta in the collapsed output file.

This step can be skipped if the analysis aims to study ribosomal RNA. Indeed, with the linkers mentioned above, collapsing allows to keep only 64 alternatives sequences (3 random nucleotides = 4^3 possibilities); since the exosome strongly binds to pre-rRNA, collapsing would lead to flattening exosome binding peaks across

pre-RNA. However, this step is essential for study of exosome binding on RNA polymerase II transcripts.

3.6.3 Alignment

Reads are then aligned to the *Saccharomyces cerevisiae* genome (SGD v64) using Novoalign (Novocraft) with genome annotation from Ensembl (EF4.74) [13], supplemented with noncoding sequences as described [14], with parameters -r Random.

```
$ novoalign -f flexbar_comp.fasta -s 1 -r Random -d  Sacchar-
omyces_cerevisiae.EF4.74.novoindex > flexbar_comp.novo
```

where Saccharomyces_cerevisiae.EF4.74.novoindex is the genome-specific index file generated by novoindex, and flexbar_comp.novo is the output file name. The "-r Unique" or "-r All" parameters are useful especially for study of exosome binding across tRNAs which share common sequences [10]. "–r" Unique will lead to preferential loss of a subset of sequences (e.g., ribosomal sequences which are represented by two identical *RDN37* sequences in the yeast reference genome).

By default, NovoAlign filters out all reads shorter than 17 nt (as shorter reads are unlikely to map uniquely to the yeast genome). For datasets obtained from Rrp44 CRAC, it was useful to align shorter sequences [15] enriched for species targeted to Rrp44 exonuclease site and bypassing the exosome channel (Rrp44 protects 9 nt while exosome + Rrp44 protects 31–33 nt). In some analyses, we then used "–l 9" parameter (instead of –l 17 default).

3.6.4 Counting Overlaps with Genomic Features

To study distribution of reads across the genome, we use pyReadCounters.py from the pyCRAC package. A GTF format file for genome annotation is required by the pyCRAC software and is critical to the interpretation of the output of the pyCRAC pipeline. pyCRAC is sensitive to the formatting within the GTF file and we find it useful to check the annotated GTF file using the pyCheckGTFfile.py command to ensure that the GTF file is suitable for use with the pyCRAC software.

```
$ pCheckGTFfile.py --gtf annotation.gtf –o annotation_checked.
gtf
```

where annotation.gtf is a GTF format file of the genome annotation.

```
$ pyReadCounters.py -f flexbar_comp.novo --gtf=annotation_ch-
ecked.gtf --rpkm
```

The output files are (1) a gtf file that can be used as input files in numerous analyses within pyCRAC package, (2) a hit table file presenting the counts of reads mapped to each genomic feature within each defined RNA class in absolute value and read number

normalized per kilobase per millions (if –rpkm parameter is specified in the command line).

3.6.5 Distribution along Genes

To observe binding distribution of exosome subunits across individual genes, we use pyPileup.py from the pyCRAC package. The output is a tab-delimited file that can be plotted to obtain a visual overview of binding along the gene of interest. This gives particularly good quality plots for RNAs that are strongly targeted by the exosome.

```
$ pyPileup.py -f flexbar_comp.novo --gtf=annotation_checked.
gtf --tab=sequence.tab -g gene.list & -r 0
```

where sequence.tab is a tab-delimited file with genes name and sequences and gene.list is a text file with the names of genes for which you want to generate output files.

-r parameter allows the user to indicate the length of flanks to be added on 5′ and 3′ ends of genes.

To study binding across a particular class of RNA, metagene plots are generated. We used custom-made scripts, still not available online. However, the computeMatrix, plotProfile, and plotHeatmap modules of the deepTools software allow for similar analyses [16].

3.6.6 Oligo-A Reads

Selection of reads containing 3′ nonencoded A tracks, allows identification of targets oligoadenylated by TRAMP prior binding of the exosome. We use custom-made scripts giving as output files (1) a fasta file containing only oligo-A reads, used for downstream analyses, (2) a text file with the ratio of oligo-A to total reads, and (3) a text file with the list of nonencoded 3′ tails.

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