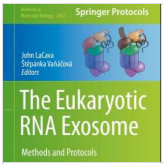

Sep 02, 2021

Cell Lysis, Two-Step Purification, and RNase Digestion

Book Chapter

In 1 collection

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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.

We present a protocol for the identification of RNA interaction sites for the exosome, using UV cross-linking and analysis 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, 4, 5, 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× 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].

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COLLECTIONS

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

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PARENT PROTOCOLS

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

MATERIALS TEXT

Yeast Strains and Culture Media

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.

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.

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.

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.

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.

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A	B	C
Illumina barcoded 5' adapter	L5Aa	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrArArGrC-OH
	L5Ab	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArUrUrArGrC-OH
	L5Ac	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrCrGrCrArGrC-OH
	L5Ad	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrGrCrUrUrArGrC-OH
	L5Ba	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArGrArGrC-OH
	L5Bb	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrUrGrArGrC-OH
	L5Bc	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrArCrUrArGrC-OH
	L5 Bd	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrCrUrCrUrArGrC-OH
	L5Ca	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrUrArGrC-OH
	L5Cb	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrGrGrArGrC-OH
	L5Cc	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArCrUrCrArGrC-OH
	L5Cd	invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrArCrUrUrArGrC-OH
Illumina 3' adapter	miRCAT 33	AppTGGAATTCTCGGGTGCCAAG/ddC/'
RT primer	miRCat RT	CCTTGGCACCCGAGAATT
PCR primers	P5_Fwd	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
	PE-miRCat_Rev.	CAAGCAGAAGACGGCATACGACCTTGGCACCCGAGAATTCC

Table 1. Oligonucleotides used in CRAC experiments

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

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.

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

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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).

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

BEFORE STARTING

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.

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 (ThermoFisher; 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.

1 **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.

Cell Lysis

2 

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.

3  2m

Add 2.5 volumes of Zirconia beads to cell pellet (~ **2.5 mL** , measure in 15 ml Falcon tubes). Vortex the cells

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00:01:00 , then put them **On ice** for **00:01:00** between each vortexing step. **Repeat five more times.**

Using a powerful vortex increases the efficiency of the lysis.

4

Add three volumes of TN150 (+ protease inhibitors) (~ **3 mL**), shake vigorously, and centrifuge the suspension in the falcon tube (**4000 x g, 4°C, 00:20:00**).

5

Transfer the supernatant (~ **4.5 mL**) to three 1.5 ml tubes and spin lysate at **16000 x g, 4°C, 00:20:00** in a microcentrifuge. Keep tubes **On ice** throughout the process.

6 Keep supernatants **On ice** , ready to be mixed with IgG sepharose beads in Step 10 below.

Protein A:IgG Purification

7

Resuspend IgG Sepharose beads by gently inverting the bottle. Pipette out **167 µl 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 TN150 lysis buffer + protease inhibitors** . Collect beads between washes by pulsing in the centrifuge to **1000 rpm** (**210 x 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.

8

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

9 Take **5 µl cleared lysate ("crude lysate")** aliquots for troubleshooting the purification, and store at **-20 °C** .

10 2h

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Mix remaining lysates with IgG Sepharose beads and rotate for a minimum of **02:00:00** at **4 °C** . This step can be extended to **Overnight** .

11

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

12 Take a **5 µl supernatant** aliquot for troubleshooting (“IgG supernatant”) and store at **-20 °C** .

13 5m

Wash beads **twice** with **10 mL TN1000** and **twice** with **10 mL TN150** For each wash, gently agitate at **4 °C** for **00:05:00** .

> Buffers used in this and all subsequent steps should **not** contain protease inhibitors.

14

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

TEV Cleavage

15

Add **20 units (U)** – **30 units (U)** of TEV protease to the beads and mix by inverting tube.

16 2h

Incubate at **18 °C** for **02:00:00** with shaking (make sure beads remain in suspension).

17

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.*

18 Take a **25 µl eluate sample** (“TEV eluate”) and store at **-20 °C** .

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RNase Digestion and Binding to Ni-NTA Resin 5m

19

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 (use 1:100 dilution in water), store it at 4 °C and use for all subsequent experiments.

20 Prepare 1.5 ml microcentrifuge tubes containing **0.4 grams (g) guanidine HCl** .

21

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

22 5m

Add **1 µl 1:100 dilution of RNace IT** (0.15 units, Agilent) to **550 µl TEV eluate** . Incubate for **00:05:00** at **37 °C** .

23 Immediately transfer the RNase treated eluate into the prepared microcentrifuge tubes containing guanidine hydrochloride. The final volume will be around **700 µl** .

24

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

25

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

26

Incubate with gentle agitation at **4 °C** **Overnight** .

27

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

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

29

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.

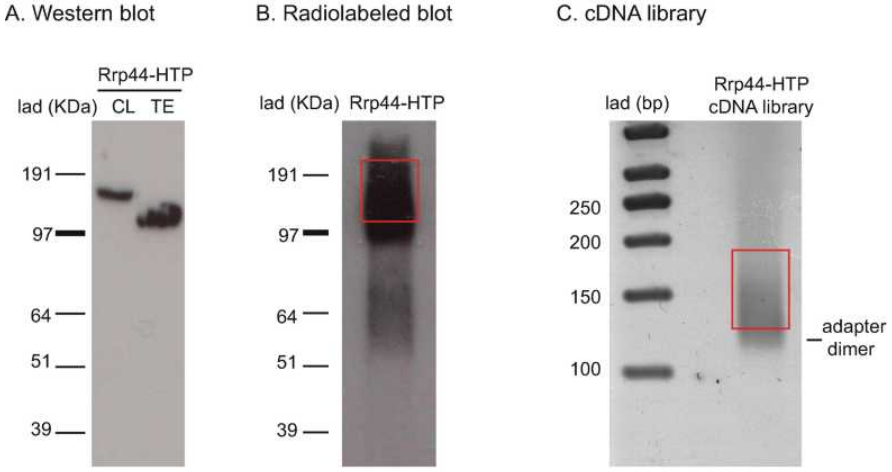

Fig. 1 Key steps of Rrp44-HTP CRAC protocol.
(**a**) Western blot to test purification of Rrp44 after lysis (Crude Lysate or CL sample) and after elution from IgG beads and TEV cleavage (TEV eluate or TE sample). Difference in size is due to cleavage of part of the tag (Protein A × 2) by TEV protease.
(**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

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