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# Linker Ligation at both Ends of RNAs on Beads

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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 cDNA (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-PreScission 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 <sup>32</sup>P (to allow RNA–protein complexes to be followed during gel separation) and linkers ligated. Note, however, that alternatives to using <sup>32</sup>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

## KEYWORDS

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

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## GUIDELINES

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× PNK buffer should be pipetted so that it rinses the side of the columns.

## 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. [ $\gamma$ <sup>32</sup>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. RNase-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.

A	B	C
Illumina 5' adapter	L5Aa	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrArArGrC-OH
	L5Ab	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrArUrArGrC-OH
	L5Ac	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrCrGrArGrC-OH
	L5Ad	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrGrCrUrUrArGrC-OH
	L5Ba	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArGrArGrC-OH
	L5Bb	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrUrGrArGrC-OH
	L5Bc	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrArCrUrArGrC-OH
	L5 Bd	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrCrUrUrArGrC-OH
	L5Ca	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrCrUrArGrC-OH
	L5Cb	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrUrGrGrArGrC-OH
	L5Cc	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrArCrUrCrArGrC-OH
	L5Cd	invddT-ACACrGrArCrGrCrUrCrUrCrCrGrArUrCrUrNrNrGrArCrUrUrArGrC-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 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.

### Dephosphorylation of RNA 3' P Ends Using Alkaline Phosphatase (TSAP)

1

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.

2




Spin out the residual 1× PNK buffer and close the column with the supplied press-on bottom stopper.

3 

To each sample, add **80 µl TSAP master mix** that contains:

- **16 µl 5× PNK buffer**
- **8 µl TSAP**
- **2 µl RNasin**
- **54 µl Milli-Q water**

4 

Mix by stirring with a pipette tip then flicking column gently.

5 

30m

Incubate at **37 °C** for **00:30:00**.

6 

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

#### On Bead Ligation of 3' miRCat-33 Linker

7

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.

8 

Spin out the residual volume of 1× PNK buffer. Close the bottom of the column with the press-on stopper.

9 

To each sample, add **76 µl miRCat master mix** containing:

- **16 µl 5× PNK buffer**
- **8 µl 10 µM miRCat-33**
- **2 µl RNasin**

-  **50 µl Milli-Q water**

10



To each sample add  **4 µl T4 RNA ligase I**.

11



4h

Incubate at  **25 °C** for  **04:00:00**.

12



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

#### Phosphorylating the 5' Ends of Cross-Linked RNA

13

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.

14







Spin out the residual volume of 1× PNK buffer. Cap the bottom of the column.

15



To the resin, add  **80 µl PNK master mix** containing:

-  **16 µl 5× PNK buffer**
-  **4 µl T4 polynucleotide kinase**
-  **56 µl Milli-Q water**
-  **4 µl 32P-ATP (10 µCi/µl)**

16



40m

Incubate at  **37 °C** for  **00:40:00**.

17



20m

Add  **1 µl 100 mM ATP** and continue the incubation for  **00:20:00**.



18 

Wash the resin **four times** with  **400 µl Wash Buffer I** and **three times** with  **400 µl 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.

#### On-Column Ligation of the 5' Adapters

19





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.

20 

Spin out the residual volume of 1× PNK buffer.

21 

Add  **75 µl 5' linker master mix** containing:

-  **16 µl 5× PNK**
-  **8 µl 10 mM ATP**
-  **2 µl RNasin**
-  **49 µl Milli-Q water**

22 

To each sample add  **1 µl barcoded 5' linker** and  **4 µl T4 RNA ligase I** .

23  

Incubate  **Overnight** at  **16 °C** .

24 

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