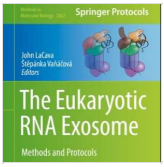

Sep 02, 2021

Generation of Library for Sequencing

Book Chapter

In 1 collection

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dx.doi.org/10.17504/protocols.io.bntimeke

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Citation: ClÃÂ©mentine Delan-Forino, David Tollervey (09/02/2021). Generation of Library for Sequencing. https://dx.doi.org/10.17504/protocols.io.bntimeke

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].

DOI

dx.doi.org/10.17504/protocols.io.bntimeke

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PROTOCOL CITATION

Clémentine Delan-Forino, David Tollervey 2021. Generation of Library for Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bntimeke

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

CREATED

Oct 22, 2020

LAST MODIFIED

Sep 02, 2021

OWNERSHIP HISTORY

Oct 22, 2020 Lenny Teytelman protocols.io

Jul 05, 2021 Emma Ganley protocols.io

Aug 24, 2021 Satyavati Kharde

Aug 26, 2021 satyavati Kharde

PROTOCOL INTEGER ID

43594

PARENT PROTOCOLS

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

GUIDELINES

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.

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

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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 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).

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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.

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.

Reverse Transcription of Purified RNA

1

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

2

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

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.

3 8m

Heat the samples to **80 °C** for **00:03:00** , then chill **On ice** for **00:05:00** . Collect the contents by brief centrifugation.

4

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

5 3m

Incubate at **50 °C** for **00:03:00** and add **1 µl SuperScript III** (Invitrogen). This step will help dissociate any nonspecifically annealed primers from the RNA.

6 1h

Incubate at **50 °C** for **01:00:00** .

7 15m

Inactivate the Superscript III by incubating the samples at **65 °C** for **00:15:00** .

8 30m

Add **2 µl RNase H** and incubate for **00:30:00** at **37 °C** .

PCR Amplification of cDNA Libraries

9

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.

10

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To **3 µl cDNA template** , add **47 µl PCR master mix** containing:

- **5 µl 10× LA Taq buffer**
- **1 µl 10 µM P5 Solexa primer**
- **1 µl 10 µM pE_miRCat reverse primer**
- **5 µl (fresh) 10 mM dNTPs**
- **0.5 µl LA TaKaRa Taq polymerase**
- **37.5 µl nuclease-free water**

We prepare three or more PCR reactions per sample to increase the complexity of our libraries.

11

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	

12 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.

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** .

13 30m

Incubate at **-20 °C** for **00:30:00** (it is better to not precipitate longer to avoid recovering too much salt).

Centrifuge at **16000 x g, 4°C, 00:30:00** .

14 Remove the supernatant and air dry the pellet. Resuspend in **15 µl MilliQ water** .

Size Selection of cDNA Libraries on Gel

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15

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.

16 Prepare a 3% Metaphor agarose gel using 1× TBE buffer (with 1:1000 SYBR Safe) and store it at **4 °C** for a minimum of **00:30:00** . 30m

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× 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.

17 

Add **5 µl 6× 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.

18 Run the gel at 80 V for approximately **02:00:00** or until the bromophenol blue dye front reaches 2 cm from the edge of the gel. 2h

19 

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.

20 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.

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

22 35m

Add **1 mL Buffer QG** from the MinElute Gel Extraction purification kit (QIAgen) and incubate the gel slices at **42 °C** for **00:15:00** – **00:20:00** to dissolve the agarose.

23

Transfer the volume to a MinElute column fitted to collection tubes and spin at **16000 x g, 00:01:00** . Discard the flowthrough.
Repeat with the leftover of buffer/agarose to bind all the sample to the same column.

24

Add **750 µl Buffer QG** and spin at **16000 x g, 00:01:00** . Discard the flowthrough.

25 10m

Add **750 µl Buffer PE** (QIAgen) to the columns and incubate for **00:10:00** at **Room temperature** . Spin at **16000 x g, 00:01:00** and discard the flowthrough.

26

Dry the columns by spinning at **16000 x g, Room temperature , 00:02:00** . Transfer the columns to clean 1.5 ml microcentrifuge tubes.

27 7m

Add **20 µl MilliQ water** on membrane and let stand for **00:02:00** – **00:05:00** . Elute the purified cDNA by spinning at **16000 x g, Room temperature , 00:01:00** .

28

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

Sequencing

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29

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.

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