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Reconstitution of the human nuclear RNA exosome

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Abstract

We describe procedures to clone, express, and reconstitute an active human nuclear RNA exosome. Individual recombinant subunits are expressed from *E. coli* and successfully reconstituted into the nuclear complex, which contains the non-catalytic nine-subunit exosome core, the endoribonuclease and exoribonuclease DIS3, the distributive exoribonuclease EXOSC10, the cofactors C1D and MPP6, and the RNA helicase MTR4.

Keywords

RNA exosome; RNA decay; Ribonuclease; Human

1. Introduction

The human nuclear RNA exosome, a multi-protein complex conserved across all eukaryotes, provides an essential platform for the processing and degradation of RNA substrates [1-3]. We previously implemented a reconstitution approach using recombinant subunits, expressed and purified from *E. coli*, to study the human exosome biochemically and structurally [4,5]. Using this strategy, our group solved an x-ray crystallography structure of the human Exo9 core, and it was determined the Exo9 scaffold is a donut-shaped structure that features a prominent central channel that is large enough to accommodate single-stranded RNA substrates [6]. The Exo9 core consists of a six-membered ring formed by the RNase PH-like proteins EXOSC4, EXOSC5, EXOSC6, EXOSC7, EXOSC8, and EXOSC9, that is capped by a three-membered ring consisting of EXOSC1, EXOSC2, and EXOSC3, which contain S1 and KH domains [6]. The RNA exosome core engages two different hydrolytic ribonucleases, DIS3 and EXOSC10, providing the enzymatic activity to cleave RNA [7,8]. Extrapolating from budding yeast x-ray structures, which revealed the exosome core engaged to the two ribonucleases [5,9-11], a similar architecture was presumed for the human system where EXOSC10 is on the “top” of the Exo9 complex and DIS3 is on the “bottom”. In point of fact, the proposed model of EXOSC10 residing on the “top” of human Exo9 core is consistent with biochemical findings that employed active human RNA exosomes purified by this reconstitution approach [5].

Cofactors have long been known to associate with the exosome to modulate cleavage activity and target specific classes of RNAs [2,3]. In human cells, immunoprecipitation studies that targeted exosome components demonstrated that the cofactor MPP6, the cofactor C1D, and the DExH RNA helicase MTR4 (MTREX/SKIV2L2) are co-purified at stoichiometric amounts [12-14]. Moreover, the combination of the Exo9 core, EXOSC10, DIS3, MPP6, C1D and MTR4 may very well be a constitutive complex in the nucleus, as RNAi targeted knock-down of EXOSC10 also decreases the level of cofactors MPP6 and MTR4 in co-immunoprecipitation experiments [13].

Purification of the two ribonucleases by themselves or in complex with the exosome core has been an outstanding challenge using human proteins. There are three DIS3 paralogs in humans: DIS3, DIS3L, and DIS3L2. Both DIS3 and DIS3L contain PIN domains [15,16], a necessary and sufficient component for interacting with the Exo9 core (as determined in the budding yeast system [17,18]). In contrast, DIS3L2 lacks the PIN domain and does not associate with the exosome core [19-24]. The Jensen and Pruijn groups demonstrated that DIS3 resides in the nucleus, while DIS3L is associated with a putative cytoplasmic exosome [15,16]. The Jensen lab attempted an approach to produce recombinant DIS3 in bacteria, but ultimately abandoned this method because the sample was heavily proteolyzed and had a low specific activity; therefore, they opted instead to characterize a semi-purified DIS3 from cultured human cells and successfully demonstrated DIS3 exoribonucleolytic activity. Purification of nuclear exosome complexes from human cells with DIS3 associated to them has also been a challenge. The most successful attempt to date was achieved by treating purified human exosome complexes with a site-specific cross-linker after affinity purification [25]; however, it should be noted this method did impact the exoribonucleolytic activity.

Human EXOSC10 has also been historically challenging to produce [4,5]. Our previous work used truncation analysis to generate protein that catalyzed a distributive exoribonucleolytic activity and interacted with the Exo9 core. In budding yeast, the PMC2NT domain of Rrp6 (human EXOSC10) has been shown to be necessary and sufficient to interact with the cofactor Rrp47 (human C1D) and allows for the purification of a more stable version of Rrp6 [11, 26-31]. In work described in this chapter, we also exploit the interaction of C1D to the N-terminal region of EXOSC10 to express and purify a stable form of EXOSC10 (although, it should be noted we still employ truncations in the C-terminal tail of EXOSC10 to obtain soluble protein). In budding yeast, the combination of the cofactors Rrp47 and Mpp6, the Exo9 core, and Rrp6 are able to interact with the Mtr4 helicase [32-34]. A similar strategy is also implemented in the human system to produce this Exo14 complex (and subcomplexes) [35]. This chapter contains approaches for cloning and protocols for expression, purification, and reconstitution of these different human subunits to form a nuclear exosome (Exo14) that is biochemically active and effectively employed for structural characterization [35]. An overall scheme for this strategy is depicted in Fig. 1.

2. Materials

2.1. Cloning

1. Human cDNA libraries from different tissues.

2. Herculase II Fusion DNA Polymerase Kit.
3. GeneArt Seamless Cloning Kit.
4. Restriction Enzymes.
5. Quickchange Site-Directed Mutagenesis Kit.
6. Standard DNA Electrophoresis Systems.
7. Vectors: pRSF-DUET, pET-DUET, pET-28, pET-21, pGEX-4T2, and pSMT3.

2.2. Protein Expression and Purification

1. ELGA Purelab Ultra 18.2 MΩ-cm ultrapure water or equivalent for all solutions.
2. 250 and 50 mL conical tubes, 1.5 mL microcentrifuge tubes.
3. Refrigerated microcentrifuge capable of 20,000 x g force (e.g. Eppendorf 5424r).
4. Luria-Bertani (LB) medium.
5. Luria-Bertani Agar plates (supplemented with antibiotics as appropriate).
6. Super Broth (SB) medium.
7. Antibiotics: 50 mg/ml kanamycin in water (0.2 μM filtered); 35 mg/ml chloramphenicol in 100% ethanol; 50 mg/ml ampicillin in water (0.2 μM filtered).
8. Bacterial strains for protein expression: *E. coli* BL21 RIL Codon Plus, BL21 (DE3) RIL Codon Plus, and One Shot BL21 (DE3) STAR.
9. Constructs of human exosome subunits as listed in Table 1.
10. Shaking incubators (18–37 °C) and 2 L baffled shaking flasks.
11. Beta-mercaptoethanol (BME).
12. Tris(2-Carboxyethyl)phosphine Hydrochloride TCEP (adjusted to pH 7.0 at 20°C with 10N NaOH and stored at –20°C for no longer than 1year).
13. Ulp1 protease (Invitrogen or purified in-house) [7] (see Note 1).
14. 5 M NaCl (0.2 μM filtered).
15. 0.25 M Isopropyl β-D-thiogalactopyranoside (IPTG)
16. 100 mM phenylmethylsulfonyl fluoride (PMSF) in 100% ethanol.
17. 2.5 M imidazole (adjusted to pH 8.0 at 20°C with concentrated HCl).
18. 10 % (v/v) IGEPAL ca-630 (in water).
19. 10 mg/ml DNase I in 20 mM Tris-HCl pH 8.0, 350 mM NaCl (aliquoted, flash-frozen and stored at –20°).
20. Tris-sucrose buffer pH 8.0: 50 mM Tris-HCl pH 8.0, 20% (w/v) sucrose (pH adjusted with NaOH; stored at 4°C).

21. Tris-sucrose buffer pH 8.6: 50 mM Tris-HCl pH 8.6, 20% (w/v) sucrose (pH adjusted with NaOH; stored at 4°C).
22. Branson 450 watt digital sonifier (or equivalent) equipped with ½ inch disrupter horn and acoustic enclosure.
23. Beckman Coulter Avanti J-26XP (or similar) equipped with JLA 8.1 and JA-20 rotors.
24. Ni wash buffer: 20 mM Tris-HCl pH 8.0, 350 mM NaCl, 10 mM imidazole, 1 mM BME (stored at 4°C).
25. Ni elution buffer: 20 mM Tris-HCl pH 8.0, 350 mM NaCl, 250 mM imidazole, 1 mM BME (stored at 4°C).
26. GST elution buffer: 20 mM Tris-HCl pH 8.0, 350 mM NaCl, 15 mM glutathione, 1 mM BME (stored at 4°C).
27. Buffer T100: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM BME (or 2 mM TCEP) (stored at 4°C) (*see Note 2*).
28. Buffer T250: 20 mM Tris-HCl pH 8.0, 250 mM NaCl, 1 mM BME (or 2 mM TCEP) (stored at 4°C).
29. Buffer T350: 20 mM Tris-HCl pH 8.0, 350 mM NaCl, 1 mM BME (or 2 mM TCEP) (stored at 4°C).
30. Buffer T1000: 20 mM Tris-HCl pH 8.0, 1 M NaCl, 1 mM BME (or 2 mM TCEP) (stored at 4°C).
31. Ni-NTA agarose resin (*see Note 3*).
32. Glutathione Sepharose 4B GST-tagged protein purification resin.
33. L-Glutathione reduced.
34. Empty, reusable columns (Bio-Rad Econo-Pac or similar).
35. 100 U/μl RNase I.
36. AKTA-FPLC (GE Healthcare) equipped with 10 mL, 1.0 mL, 0.5 mL and 0.4 mL injection loops, gel filtration columns (HiLoad 26/60 Superdex 75 prep-grade, HiLoad 26/60 Superdex 200 prep-grade, Superdex 200 Increase 10/300 GL, Mono S 10/100, Mono Q 10/100), and heparin affinity column (HiTrap Heparin HP 5 mL). All columns are kept in a cold room or refrigerator at 4°C.
37. Ultra centrifugal filter devices with 10K and 30K molecular weight cutoffs (0.5 mL, 4.5 mL and 15 mL capacities; for example Amicon, Millipore).
38. Bradford reagent
39. NanoDrop 2000 Spectrophotometer (Thermo Scientific) or equivalent.
40. 0.2 μm syringe filter (e.g. Acrodisc Syringe Filter, 25 mm (Pall Corporation)).

41. NuPAGE system (Invitrogen) or comparable system for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).
42. MOPS running buffer.
43. 4–12% gradient polyacrylamide Bis-Tris gels.
44. 4x LDS (Lithium Dodecyl Sulfate) sample dye (adjusted to 5% BME prior to use).
45. Gel staining boxes.
46. Protein ladder for SDS-PAGE, 10–200 kDa range (e.g. Benchmark, Thermo-Fisher).
47. Coomassie stain solution: 40% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie R250.
48. Destain solution: 10% (v/v) methanol, 10% (v/v) glacial acetic acid.
49. Gel imaging apparatus with UV and white light illumination (Bio-Rad Gel doc or similar).

2.3. Exo9, Exo12, Exo13, and Exo14 reconstitution

1. Dialysis cassettes (3–12 mL volume and 0.5 mL volume 7000 kDa MW cutoff, Thermo-Fisher Slide-A-Lyzer and Thermo-Fisher Slide-A-Lyzer G2 or similar).
2. Buffer R100a: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM BME.
3. Buffer R100b: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5 mM MgCl₂, 2 mM TCEP.
4. Buffer R50a: 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM BME.
5. Buffer R50b: 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5 mM MgCl₂, 2 mM TCEP.

3. Protocols

3.1. Cloning

In the interest of preparing RNA exosome subunits and cofactors in the absence of potential contaminating cofactors, human RNA exosome subunits are expressed and purified from recombinant bacterial hosts. Here, we will briefly summarize our strategies for generating these constructs without providing specifics on protocols as these individual steps should be readily adaptable to any preferred cloning strategy commonly used in laboratories. All constructs are summarized in Table 1.

Most of the human exosome proteins could be obtained as single homogeneous polypeptides; however, in the case of the RNase-PH like subunits and EXOSC10, it was necessary to coexpress obligate heterodimeric and heterotrimeric combinations to maintain solubility during purification [5,6]. In order to produce near native N-termini, human exosome proteins were engineered with N-terminal expressed cleavable fusion proteins (i.e.

Smt3 and GST fusions). Coding DNA for each of the human exosome genes (EXOSC1-9, DIS3) and cofactors (C1D, MPP6, MTR4/SKIV2L2) was amplified by PCR from a human placental cDNA library. Human EXOSC10/RRP6 was codon-optimized for expression in *E. coli* and synthesized using the GeneArt platform (Invitrogen). All sequences were analyzed by DNA sequencing for fidelity after integration into the target backbone.

3.1.1. Cloning of Human RNasePH-like domains: EXOSC9/EXOSC4

heterodimer, EXOSC7/EXOSC6/EXOSC8 heterotrimer, EXOSC5—Genetic sequences encoding human EXOSC9 and EXOSC4 were inserted into MCS1 and MCS2 of plasmid pDUET-1, respectively (Table 1). Plasmid pETDUET-EXOSC9(MCS1)/EXOSC4(MCS2) encodes two polypeptides, an N-terminal His-tagged EXOSC9 fusion and untagged EXOSC4. This plasmid was used to transform *E. coli* BL21 (DE3) Codon Plus RIL. DNA sequences encoding human EXOSC7 and EXOSC6 were inserted into a pRSF-DUET-1 vector that is modified to express a Smt3 fusion within the MCS1. EXOSC7 was placed into MCS1 and EXOSC6 was placed into MCS2. Plasmid pRSF-DUET-His₆-Smt3-EXOSC7(MCS1)/EXOSC6(MCS2) encodes two polypeptides, an N-terminal His-tagged Smt3 EXOSC7 fusion and untagged EXOSC6. A second plasmid pET21-Smt3-EXOSC8 was prepared that places EXOSC8 within the MCS of a pET21a-Smt3 vector, resulting in a Ulp1 cleavable Smt3-EXOSC8 fusion polypeptide (without a His tag). The resulting two plasmids were co-transformed into *E. coli* BL21 (DE3) STAR cells to express the heterotrimer. DNA encoding human EXOSC5 was cloned into pSmt3-Topo generating a His₆-tagged fusion construct (pSmt3-EXOSC5) used for transformation into *E. coli* BL21 (DE3) Codon Plus RIL.

3.1.2. Human exosome S1/KH domain containing subunits: EXOSC2,

EXOSC3 and EXOSC1—PCR DNA encoding human EXOSC2, EXOSC3, or EXOSC1 was inserted into MCS1 of a modified version of the pRSF-DUET-1 plasmid to generate plasmids pRSF-DUET-His₆-Smt3-EXOSC2, pRSF-DUET-His₆-Smt3-EXOSC3, and pRSF-DUET-His₆-Smt3-EXOSC1, respectively. These expression constructs encode in-frame polypeptide fusions that contain an N-terminal hexahistidine-tagged Smt3 fusion followed by the respective exosome polypeptides. These plasmids were used to transform *E. coli* BL21 (DE3) Codon Plus RIL.

3.1.3. Cloning of Human DIS3—Human DIS3 cDNA was inserted into the pGEX-4T2

expression vector. This expression construct encodes an in-frame polypeptide fusion of a thrombin cleavable N-terminal GST tag followed by wild-type DIS3. Quick Change Mutagenesis was employed to make mutant proteins DIS3 (D146N, D487N) and DIS3 (D487N), in order to mutate the metal binding residues for disruption of the endoribonucleolytic and exoribonucleolytic active sites, respectively [7]. Each of the plasmids was used to transform *E. coli* BL21 Codon Plus RIL for protein expression.

3.1.4. Cloning of Human EXOSC10/C1D heterodimer—*H. sapiens* EXOSC10/

RRP6 (PM/SCL-100) is very unstable as a full-length protein. Therefore, our lab has employed multiple strategies with limited success [5,4]. To achieve high quantities of human EXOSC10, we adopted a strategy employing an EXOSC10 sequence commercially

optimized for expression in *E. coli* (GeneArt, Invitrogen) and expressing EXOSC10 with its obligate binding partner C1D [13-15]. The EXOSC10 gene was inserted into MCS1 of a pRSF-DUET-His₆-Smt3 vector. C1D was generated from a cDNA library and subcloned into MCS2 of the pRSF-DUET-His₆-Smt3-EXOSC10 vector using custom primers and Gibson assembly cloning. EXOSC10 expression levels and protein stability were further optimized by introducing an internal deletion of amino acids 649-704 (replaced by a linker sequence SRGSGSGSGSGS) and by truncating the EXOSC10 C-terminus at amino acid 804 yielding the pRSF-DUET-His₆-Smt3-EXOSC10 1–804 649–704/C1D construct. Point mutations at D313N to disrupt the exoribonucleolytic active site of EXOSC10 were introduced using custom-made primers for Gibson assembly [9,12]. BL21 (DE3) Codon Plus RIL STAR cells were transformed with the EXOSC10 1–804 649–704 /C1D heterodimer construct.

3.1.5. Cloning of Human Exosome cofactors: MPP6 and MTR4 (MTREX/SKIV2L2)—The full-length *H. sapiens* M-Phase-Phosphoprotein 6 (MPP6) gene was PCR-amplified from placenta cDNA pool, digested with appropriate restriction enzymes and subcloned into pSmt3-TOPO yielding a His₆-Smt3-MPP6 fusion construct. The helicase MTR4 was amplified from human kidney cDNA and inserted into pET-28a with an N-terminal decahistidine-Smt3 tag. These plasmids were used to transform *E. coli* BL21 (DE3) Codon Plus RIL.

3.2. Protein Expression

3.2.1. Cell Growth and Expression of Human Exo9 core subunits (EXOSC9/EXOSC4, EXOSC7/EXOSC8/EXOSC6, EXOSC5, EXOSC2, EXOSC3, EXOSC1), EXOSC10 1–804 649–704 /C1D, DIS3, MTR4, and MPP6

1. Inoculate one or two 100 mL flasks of LB containing appropriate antibiotics with frozen bacteria from a glycerol stock and grow overnight, shaking at 37°C. For human DIS3, freshly transform the expression vector into BL21 CodonPlus-RIL (Stratagene) with 50 µg/mL of ampicillin and 35 µg/mL chloramphenicol. Use one colony to inoculate 250 mL over night LB culture at 37°C in a 500 mL shaker flask with antibiotics (*see Note 4*).
2. Inoculate 10-20 shaker flasks of 1 L Super Broth with 10 mL of the LB overnight culture and appropriate antibiotic and grow at 37°C to an OD₆₀₀ of 1–2. When the OD₆₀₀ of the culture reaches ~1.8, transfer flask to an ice bath for ~30 minutes and add 20 mL of ethanol to a final concentration of 2%. Table 1 indicates the specific amounts of culture needed and total yields expected for each protein.
3. Induce protein expression by adding IPTG to a final concentration of 0.25 mM (1 mL of 250 mM stock) and return flasks to an incubator set at 18°C, overnight.
4. Harvest cells by centrifugation at 4000 × g (Beckman JLA-8.1) for 20 minutes at 4°C and discard cleared supernatant.
5. Resuspend cell pellets in Tris-sucrose buffer pH 8.0 at a concentration of ~0.5 g cell wet weight per mL. Unlike all other subunits, DIS3 needs to be resuspended in Tris-sucrose buffer pH 8.6 which increases the yield of soluble protein.

6. Distribute resuspended pellets into 50 mL conical tubes, snap-freeze in liquid nitrogen and store at -80°C .

3.3. Protein purification

Below are protocols for preparation of 5–50 mg (final yield) of purified exosome components. Sonication is performed at low power with constant stirring on ice/water slurry in the presence of the protease inhibitor PMSF.

3.3.1. Purification of Exo9 Core Components EXOSC1, EXOSC2, and EXOSC3, EXOSC7/EXOSC6, EXOSC5/EXOSC8 and EXOSC4/EXOSC9 and the cofactor MPP6

1. Thaw all conical tubes of cell suspension from one round of culture in room temperature water. Depending on volume, adjust the final volume to 200 mL or 2 x 200 mL with corresponding Tris-Sucrose buffer whilst adding 14 mL 5 M NaCl, 0.8 mL 2.5 M imidazole, 14 μL 14.3 M BME, 2 mL 100 mM PMSF, 2 mL 10% IGEPAL ca-630, 200 μL DNase I (10 mg/mL). The final buffer composition will be 50 mM Tris pH 8.0, 20% sucrose, 350 mM NaCl, 10 mM imidazole, 1 mM BME, 1 mM PMSF, 0.1% (v/v) IGEPAL ca-630, 10 $\mu\text{g/mL}$ DNase I.
2. On ice/water slurry with rapid stirring, sonicate at 65 % output for 3 x 2 min with 1 s on 3 s off to disrupt cells.
3. Pellet cell debris for 45 minutes at 44,000 x g in a JA-20 rotor (or similar) at 4°C (see Note 5).
4. Equilibrate 5–10 mL of Ni-NTA resin with 3 x 1 volume of Ni wash buffer at 4°C .
5. Transfer resin to one to two 250 mL conical tubes and apply supernatant.
6. Slowly rotate for 10–30 min.
7. Transfer the resin to a disposable column and allow the lysate to pass by gravity flow.
8. Wash resin with ~200 mL Ni wash buffer.
9. Elute protein with 10 mL of Ni elution buffer (see Note 6).
10. Add 1:1000 mass ratio of Ulp1 and incubate at 4°C overnight for all Smt3 containing samples (see Notes 7–9).
11. Filter the eluate through a 0.2 μm syringe filter and inject over Superdex 200 pg (for EXOSC3, EXOSC4/EXOSC9, EXOSC6/EXOSC7/EXOSC8 and MPP6) or Superdex 75 pg (for EXOSC1, EXOSC2, and EXOSC5) columns that have been equilibrated with buffer T350 (see Note 10). The Superdex 200 elution profile is shown for MPP6 as it is the most challenging of these subunits (Fig. 2C) (see Note 11). Collect 5 mL fractions.
12. Perform SDS-PAGE analysis of peak fractions (see Notes 12–14).

13. Prepare 'Fractions' samples by mixing 30 μ L of each fraction with 10 μ L of 4x LDS loading dye.
14. Run 2.5–15 μ L of each sample on a 12-well, 4–12% acrylamide Bis-Tris gel in MOPS-SDS running buffer (180 V limiting, 50 min, room temp).
15. Transfer the gel to a staining box and stain by gently shaking in Coomassie stain solution for 30 min.
16. Discard the stain solution in the waste, rinse the gel, and fill with destain solution. Shake for 2-3 hrs and view on gel imaging system.
17. Concentrate each of the peak fractions to 5–10 mg/mL in a 15 mL capacity Amicon Ultra 15 (30K MWCO) concentrator at 3,000 x g in a hanging bucket rotor (*see* Note 15). Use an Amicon Ultra 15 10K MWCO concentrator for EXOSC1 and EXOSC5.
18. Aliquot, flash freeze, and store at -80°C for later use.

3.3.2. Purification of EXOSC10 1–804 649–704 /C1D, and MTR4

1. Perform steps 1–9 of Subheading 3.3.1.
2. Elute EXOSC10 1–804 649–704 /C1D with 10 mL Ni elution buffer and immediately proceed to Subheading 3.3.2 step 4 without Ulp1 digestion (*see* Note 16).
3. Elute MTR4 with Ni elution buffer. Add 1:1000 ratio (by mass) of Ulp1 and incubate over night at 4°C .
4. Filter sample (either EXOSC10 1–804 649–704 /C1D or MTR4) through a 0.2 μ m syringe filter and inject over a HiLoad 26/600 SD200 prep-grade column that has been equilibrated with buffer T350.
5. Collect the peak fractions (Fig. 2).
6. Perform SDS-PAGE analysis of peak fractions (refer to Subheading 3.3.1 steps 12–16).
7. If required, concentrate peak fractions using a 15 mL Amicon Ultra 15 (30K MWCO) at 3,000 x g in a hanging bucket rotor to a volume of $\sim$ 10 mL (*see* Note 17).
8. Transfer sample to a dialysis cassette and perform overnight dialysis at 4°C into 2 L of buffer T250 for EXOSC10/C1D or buffer T100 for MTR4.
9. Filter sample (either EXOSC10 1–804 649–704 /C1D or MTR4) through 0.2 μ m and inject over a HiTrap Heparin column that has been equilibrated with the corresponding dialysis buffer (*see* Note 18).
10. Elute with a linear gradient to 100% Buffer T1000 over 20 column volumes (Fig. 3, top panel).

11. Collect 5 mL fractions for the flow-through and 3 mL fractions for the gradient elution.
12. Collect peak fractions (Fig. 3, bottom panel) and concentrate to >10 mg/mL in a 15 mL Amicon Ultra 15 (30K MWCO) at 3,000 x g in a hanging bucket rotor.
13. Aliquot, flash freeze, and store at -80 °C for later use.

3.3.3. Purification of DIS3

1. Thaw cell suspension in room temperature water. On ice, adjust the volume to 200 mL or 2 x 200 mL with corresponding Tris-Sucrose buffer whilst adding 14 mL 5 M NaCl, 0.8 mL 2.5 M imidazole, 14 µL 14.3 mM BME, 2mL 100 mM PMSF, 2 mL 10% (v/v) IGEPAL ca-630, 200 µl DNase I (10 mg/mL). The final buffer composition will be 50 mM Tris pH 8.6, 20% sucrose, 350 mM NaCl, 10 mM imidazole, 1 mM BME, 1 mM PMSF, 0.1% (v/v) IGEPAL ca-630, 10 µg/mL DNase I.
2. On ice/water slurry with rapid stirring, sonicate at 65% output for 3 x 2 minutes with 20% duty cycle (e.g. 1 s on, 4 s off, 120 repeats) to disrupt cells.
3. Distribute lysate to centrifuge tubes and pellet cell debris for 45 minutes at 44,000 x g in a JA-20 rotor.
4. Preequilibrate 5 mL of Glutathione Sepharose 4B resin (GE Biosciences) with Buffer T350 (pH adjusted to 8.0).
5. Transfer resin to a 250 mL conical tube and apply supernatant.
6. Rotate with very gentle agitation for 60 min.
7. Transfer the resin to a disposable column and allow the lysate to pass by gravity flow.
8. Wash resin with ~200 mL Buffer T350 (pH adjusted to 8.0).
9. Elute GST-DIS3 with 10 mL of GST elution buffer.
10. Measure GST-DIS3 by Bradford assay. Add 1 unit of thrombin per mg eluted protein and incubate at 4°C overnight (see Note 19).
11. Filter the cleaved DIS3 through a 0.2 µm syringe filter and inject the entire ~10 mL sample onto a HiLoad 26/600 Superdex200 that has been equilibrated with buffer T350. Collect 5 mL fractions (Fig. 2).
12. Pool peak fractions, if required concentrate using a 15 mL Amicon Ultra 15 (30K MWCO) at 3,000 x g in a hanging bucket rotor to a volume of ~10 mL and transfer to 12 mL dialysis cassette (see Note 20).
13. Dialyze peak fractions overnight in 2 L of T100 buffer.
14. Inject peak fractions from SD200 onto a monoQ 10/100 column preequilibrated with T100 buffer.

15. Collect 5 mL fractions for the flow through and 3 mL fractions for the gradient elution.
16. Elute with a linear gradient to 40% Buffer T1000 over 20 column volumes (Fig. 4).
17. Concentrate peak fractions to >10 mg/mL in a 15 mL Amicon Ultra 15 (30K MWCO) at 3,000 x g in a hanging bucket rotor
18. Aliquot, flash freeze, and store at -80 °C for later use (*see* Note 21).

3.4. Reconstitution of nuclear human exosome and subcomplexes

Previous reconstitution strategies have been successful in the analysis of nuclear forms of the budding yeast RNA exosome; a similar strategy is described here that first assembles and purifies a 9-subunit core that comprises the six RNase PH-like subunits with the three S1/KH cap components, and then subsequently the active components DIS3 and EXOSC10 1–804 649–704 /C1D and cofactors MPP6 and MTR4. The entire nuclear exosome complex is purified by gel filtration chromatography. We can isolate complexes of the Exo9 core, Exo12, Exo13, and Exo14 with apparent subunit stoichiometry (*see* Note 22).

3.4.1. Reconstitution of human Exo9 core

1. Thaw aliquots of purified EXOSC1, EXOSC2, EXOSC3, EXOSC4/EXOSC9, EXOSC5, and EXOSC6/EXOSC7/EXOSC8 proteins and protein subcomplexes and place on ice immediately after the last piece of ice disappears.
2. Centrifuge proteins at 20,000 x g, 4°C for 1 min to pellet any material that had precipitated after freeze-thawing.
3. Measure protein concentration of the supernatants.
4. Mix the appropriate quantities of core subunits as detailed in Table 2 in T350 Buffer at ~3–5 g/L in a volume of 5–10 mL. Higher concentrations will lead to the production of insoluble aggregates during dialysis. Incubate on ice for 30–45 minutes.
5. Inject the resulting mixture into a Slide-A-Lyzer dialysis cassette (MWCO 7000) and dialyze at 4°C for at least 6 hours in 2 L of R100a buffer. Switch the cassette to 2 L of R50a buffer and dialyze overnight at 4°C.
6. Remove insoluble material by passing the reconstituted mixture through a 0.2 µm filter.
7. Load sample onto a gel filtration column (Superdex 200 26/60, GE Biosciences) preequilibrated with R50a Buffer and collect 5 mL fractions. The complex elutes as a monodisperse peak (Fig. 5A).
8. Analyze peak fractions by SDS-PAGE loading 5–15 µl per well (4–12% Nu-PAGE gels and Coomassie staining as described in Subheading 3.3.1. step 12) and pool fractions containing stoichiometric amounts of core subunits.

9. Apply sample to a Mono S 10/100 (GE Biosciences) equilibrated with R50a. The 9-subunit human core complex elutes from the Mono S ion exchange column by applying a linear gradient of NaCl over 20 column volumes from 50 mM NaCl to 600 mM NaCl, eluting at approximately 300 mM NaCl [6]. Collect 3 mL fractions (Fig. 5B) (*see* Note 23).
10. Collect the peak fractions (Fig. 5) and concentrate in a 15 mL capacity Amicon YM-30 (3,000 x g, 15 min spins in a hanging bucket rotor) to ~10 mg/mL. Expected overall yield for human core is around 5 mg.
11. Aliquot, flash freeze, and store at -80°C for later use.

3.4.2. Exo12, Exo13, and Exo14 reconstitutions—Each of the reconstitutions for Exo12, Exo13 and Exo14 are performed in a similar manner. The difference between these three reconstitutions is the identity of proteins that are present in the initial mixture of components. It should also be noted that there are slight variations of the buffers used as compared to the Exo9 reconstitution. The Exo12 reconstitution consists of core, MPP6, and MTR4. Exo13 consists of core, MPP6, and EXOSC10 1–804 649–704 /C1D. Exo14 consists of core, MPP6, EXOSC10 1–804 649–704 /C1D, MTR4, and DIS3.

1. Thaw aliquots of purified DIS3, MPP6, MTR4, and His₆-Smt3-EXOSC10 1–804 649–704 /C1D proteins and reconstituted Exo9 core (in your hand to warm quickly) and place on ice immediately after the last piece of ice disappears.
2. Centrifuge proteins at 20,000 x g, 4°C for 1 min to pellet any material that had precipitated after freeze-thawing.
3. Measure protein concentration of the supernatants.
4. Incubate the prepared human Exo9 core with excess amounts of cofactors and active components (for sample reconstitutions, *see* Tables 3, 4 and 5) to reconstitute Exo12, Exo13, and Exo14 complexes (*see* Note 24). Reconstitutions are performed in T350 buffer with a total protein concentration of ~10 mg/mL. Volumes are typically between 200 to 500 µL.
5. For reconstitutions that contain His₆-Smt3-EXOSC10 1–804 649–704 /C1D, add the Ulp1 protease at a mass ratio of ~1:100 relative to His₆-Smt3-EXOSC10 1–804 649–704 /C1D, mix by gently pipetting up and down once and incubate on ice for 45 minutes.
6. Place sample in a Slide-A-Lyzer G2 0.5 mL 7000 MWCO dialysis cassette and dialyze in two steps against reconstitution buffer at 4°C. First into 2 L R100b buffer at least 4 hours, second into R50b buffer for at least 12 hours (*see* Notes 25 and 26).
7. Remove dialysate from dialysis cassette, transfer to a 1.5 mL tube and spin for 1 minute at maximum speed in a chilled table top centrifuge.
8. Apply sample to a Superdex 200 Increase 10/300 G1 column (GE Healthcare) preequilibrated with buffer R50b.

9. Perform SDS-PAGE analysis (as describe in Subheading 3.3.1. step 12) to determine fractions containing stoichiometric amounts of each subunit (Figure 6).
10. Pool fractions and concentrate to 10–20 mg/mL using 0.5 mL Amicon Ultra 30K MWCO centrifugal filters (Merck Millipore Ltd), snap-freeze in liquid nitrogen, and store at –80°C. Total expected yield per reconstitution of Exo14 is 400–500 µg.

4. Notes

1. *S. cerevisiae* Ulp1 residues 403–621 can be purchased commercially or generated in house [36]. Ulp1 cleaves N-terminal Smt3 tags after Gly98 of Smt3.
2. For routine protein purification we generally use BME as a reducing agent as it is much cheaper. We prefer TCEP for purification using affinity columns as BME can often lead to distortion of the UV curve during gradient runs. As TCEP displays superior stability it is also implemented for later steps in the reconstitution protocols.
3. As a cost-saving measure, we routinely strip and regenerate our nickel resin according to the manufacturer's protocol. Used Ni resin is stored in 20% (v/v) ethanol at 4°C.
4. All expression strains (except DIS3) can be stored at –80°C at 25% glycerol (v/v) and are stable for years. DIS3 needs to be transformed and freshly plated each time.
5. Many of the proteins are prone to protein degradation if left in the lysates for extended periods. Therefore, cell lysates should be spun down and immediately applied to affinity resins to minimize proteolysis.
6. Batch elution followed by measurement of protein concentration by Bradford assay can be performed here using larger volumes (e.g. 24 mL; eluting 8 x 3 mL). This allows for pooling of high protein fractions only but may necessitate further concentration to a volume suitable for injection onto gel filtration columns (i.e. 10 mL).
7. Do not mix samples and do not place the samples on rotisseries for incubation with Ulp1 protease. We have detected precipitation of the protein preparations with agitation.
8. For EXOSC3 and MPP6 we add ~100 U RNase I/25mg protein at this step. These proteins are prone to multimerization/aggregation and we observe higher yields of the monomers if RNA is digested. RNase I can be easily removed from the preparation during gel filtration and does not interfere with downstream assays. RNase A on the other hand may be carried over during downstream steps and should be avoided.

9. EXOSC3 is prone to incomplete Ulp1 digest. SDS-PAGE analysis (described in Subheading 3.3.1 step 12) should be performed prior to gel filtration to confirm cleavage is complete. Otherwise, add additional Ulp1 and RNase I.
10. Cross-contamination of inactive complexes with active ribonuclease, in particular EXOSC10 which is “sticky” is a real concern. We routinely inject one loop volume of 4 M guanidine-hydrochloride over our columns during the column equilibration step to remove contaminants.
11. MPP6 is only about ~25–50% pure. A fact that needs to be taken into consideration during reconstitution of Exo12, Exo13, and Exo14 complexes. This purification step is an instance where further optimization may be necessary.
12. SDS-PAGE analysis is recommended for all subunit preparations, however, it is crucial for cases where multiple subunits are copurified (i.e. EXOSC7/EXOSC8/EXOSC6 and EXOSC4/EXOSC9) to ensure only fractions containing stoichiometric amounts of protein are selected.
13. Some subunits elute in multiple peaks. We generally collect the final peak containing the desired protein under the assumption that it is monomeric.
14. In most cases of purified subunits, there is trace amount of cleaved Smt3 contamination. This is not a significant issue, as it will be removed during later reconstitution and purification steps. However, if Smt3 is present at levels that contribute to the overall protein concentration, this will impact the measurement of protein concentration by Bradford assay. Therefore, it will be important to increase the amount of total material of this subunit preparation during reconstitution to correct for the contribution of Smt3 to the protein concentration measurement.
15. Make sure to mix the resulting concentrate to fully recover material from the concentrator.
16. For catalytically inactive EXOSC10 we routinely add RNase I at 100U /25mg of protein at this step (also see Subheading 3.3.1 step 10).
17. This is to facilitate injection using a 10 mL injection loop for subsequent Heparin affinity purification. Alternatively, multiple, sequential injections of larger volumes or use of a sample pump (e.g. S9 for AKTA system) can be employed.
18. Binding capacity for 5 mL HiTrap Heparin columns according to manufacturer is estimated at 15 mg antithrombin II total. We have successfully loaded and recovered higher protein concentrations but recommend use of multiple 5 mL columns in sequence with protein loads that exceed > 20 mg.
19. GST-DIS3 is extremely prone to precipitation, therefore avoid shaking and do not use a rotisserie (or other mixing device) while incubating with thrombin. For catalytically inactive DIS3, we add 100 U RNase I/25 mg protein.

20. For Heparin affinity purification, sequential injections can be performed instead of concentration of the sample to a smaller volume.
21. Yields should usually be at 20 mg or more per 18 L of bacterial culture. Occasionally, however, we experience unexplained instances of low yields in the hands of all our users. In this case, we recommend starting anew with DIS3 transformation and culture.
22. As in earlier steps, regular cleaning of columns with one injection loop volume of 4 M guanidine-hydrochloride during equilibration is recommended to prevent cross-contamination with active ribonuclease. If several complexes are prepared on the same day, run inactive complex first.
23. This step is crucial to remove contaminating nucleases copurified from bacterial lysate.
24. MPP6 has a purity of ~25–50% and is prone to aggregation; therefore a sevenfold to eightfold excess is added for reconstitutions of Exo12, Exo13, and Exo14.
25. Addition of 0.5 mM MgCl₂ is meant to satisfy the metal binding sites of EXOSC10 and DIS3.
26. We prefer the use of TCEP as a reducing agent during reconstitutions due to its superior stability. However, reconstitution steps could be performed with Buffers R50a and R100a, which contain the reducing agent BME, as a more inexpensive option.

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Fig. 1. Reconstitution and purification overview for human RNA exosome Exo14 complex.
 (Left to Right) Schematic depiction of individual subunits and subunit combinations that are used to reconstitute the Exo9 core. The resulting Exo9 core is then added to EXOSC10/C1D, DIS3, MPP6, and MTR4 to form the full nuclear Exo14 complex.

Fig. 2.

Gel filtration purification of (A) DIS3, (B) MTR4, (C) MPP6, and (D) EXOSC10 1-804 649-704 /C1D. MTR4, and MPP6 samples were each digested with Ulp1 prior to loading onto S200 columns. DIS3 was digested with thrombin prior to loading S200 column. Traces show absorbance at 280 nm, and Coomassie-stained gels of fractions near to and including the main peak are shown below.

Fig. 3.
Heparin affinity purification for (A) His₆-Smt3-EXOSC10 1-804 649-704 /C1D and (B) Ulp1 cleaved MTR4. The (Top) traces show absorbance at 280 nm (A_{280}) (black line) and conductivity (gray dashed line), and (Bottom) Coomassie-stained gels of load and fractions that flank and contain the main peak are shown below.

Fig. 4. Anion exchange purification of DIS3.

(Top) Traces show absorbance at 280 nm (A_{280}) (black line) and conductivity (gray dashed line), and (Bottom) Coomassie-stained gels of the load and fractions near to and including the main peak are shown below.

Fig. 5. Reconstitution and purification of human 9 component exosome (Exo9).

(A) A_{280} trace and Coomassie-stained SDS-PAGE analysis of fractions from SD200 PG gel filtration of the Exo9 reconstitution. (B) A_{280} trace and Coomassie stained SDS-PAGE analysis of fractions from anion exchange (Mono Q) purification of the Exo9 reconstitution. Bands for EXOSC3 and EXOSC2 overlap. Complete separation of subunits can be achieved by extended gel run times and loading of small volumes.

Fig. 6. Reconstitution and purification of human Exo12 (A) Exo13 (B) and Exo14 (C).
A₂₈₀ traces and Coomassie-stained SDS-PAGE analysis of fractions from gel filtration of each indicated reconstitution.

Table 1:

Summary of expression vectors and purification techniques for human nuclear exosome components

Protein	Vector	<i>E. coli</i> Strain	Antibiotic	Amount of Liquid Culture	Columns	Expected Yield
EXOSC1	pRSF-DUET-His ₆ -Smt3-EXOSC1	BL21-DE3-CP	Kan+ Chlor+	6 L	MAC S75	50 mg
EXOSC2	pRSF-DUET-His ₆ -Smt3-EXOSC2	BL21-DE3-CP	Kan+ Chlor+	6 L	MAC S75	50-100 mg
EXOSC3	pRSF-DUET-His ₆ -Smt3-EXOSC3	BL21-DE3-CP	Kan+ Chlor+	12 L	MAC S200	20-30 mg
EXOSC5	pSmt3-TOPO-EXOSC5	BL21-DE3-CP	Kan+ Chlor+	6 L	MAC S75	50 mg
EXOSC6/ EXOSC7/ EXOSC8	pET21a-Smt3-EXOSC8 pRSF-DUET- His ₆ -Smt3- EXOSC7 (MCS1)-EXOSC6 (MCS2)	BL21-Star	Kan+ Amp+	18 L	MAC S200	20 mg
EXOSC4/ EXOSC9	petDUET-His ₆ -Smt3- EXOSC9 (MCS1)-EXOSC4 (MCS2)	BL21-DE3-CP	Amp+ Chlor+	12 L	MAC S200	25 mg
MPP6	pSmt3-TOPO-MPP6	BL21-DE3-CP	Kan+ Chlor+	18 L	MAC S200	4-5 mg
DIS3	pGEX4T2 -DIS3	BL21-CP	Amp+ Chlor+	18 L	Glutathione Sepharose S200 monoQ	20 mg
EXOSC10 1– 804 649– 704 /C1D	pRSF-DUET-His ₆ -Smt3- EXOSC10 1–804 649–704 (MCS1)-C1D (MCS2)	BL21-DE3- CPRIL STAR	Kan+ Chlor+	18 L	MAC Heparin S200	30 mg
MTR4	pET28-His ₁₀ -Smt3-MTR4	BL21-DE3-CP	Kan+ Chlor+	18 L	MAC Heparin S200	20 mg

Table 2:

Sample Exo9 reconstitution

Component	Molecular weight (Da)	mg/mL	μ M	Multiplier	nmol needed	μ L needed
EXOSC1	23,065	19.2	830	2.5	250	300
EXOSC2	34,337	15.1	440	1.5	150	341
EXOSC3	31,185	15.5	500	2.0	200	402
EXOSC5	25,449	24.9	980	1.5	150	153
EXOSC6/EXOSC7/EXOSC8	91,921	11.6	126	1	100	792
EXOSC4/EXOSC9	68,000	10.4	153	1.5	150	981
Buffer T350						7.0 (mL)

Table 3:

Sample Exo12 reconstitution

Component	Molecular weight (Da)	mg/mL	μ M	Multiplier	nmol needed	μ L needed
Exo9	273,957	17.3	63	1	4.2	66.5
DIS3	109,058	25.9	240	2	8.4	35.4
MPP6	19,000	12.6	660	7.5	31.5	48
MTR4	117,805	23.9	200	2	8.4	41.4
Buffer T350						168

Table 4:

Sample Exo13 reconstitution

Component	Molecular weight (Da)	mg/mL	μ M	Multiplier	nmol needed	μ L needed
Exo9	273,957	17.3	63	1	3.2	50.7
His ₆ -Smt3-EXOSC10 1-804 649-704 /C1D	116,633	24.5	210	4	12.8	60.9
DIS3	109,058	25.9	240	2	6.4	26.9
MPP6	19,000	12.6	660	7.5	24.3	36.8
Ulp1	26,000	3	120			6
Buffer T350						175

Table 5:

Sample Exo14 reconstitution

Component	Molecular weight (Da)	mg/mL	μ M	Multiplier	nmol needed	μ L needed
Exo9	273,957	17.3	63	1	3.5	55.4
His ₆ -Smt3-EXOSC10 1-804 649-704 /C1D	116,633	24.5	210	4	14	66.6
DIS3	109,058	25.9	240	2	7	29.2
MPP6	19,000	12.6	660	7.5	26.3	39.8
MTR4	117,805	23.9	200	2	7	34.5
Ulp1	26,000	3	120			6
Buffer T350						234