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Purification and Characterization of Human DNA Ligase IIIα Complexes After Expression in Insect Cells

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Abstract

With improvements in biophysical approaches, there is growing interest in characterizing large, flexible multi-protein complexes. The use of recombinant baculoviruses to express heterologous genes in cultured insect cells has advantages for the expression of human protein complexes because of the ease of co-expressing multiple proteins in insect cells and the presence of a conserved post-translational machinery that introduces many of the same modifications found in human cells. Here we describe the preparation of recombinant baculoviruses expressing DNA ligase IIIα, XRCC1, and TDP1, their subsequent co-expression in cultured insect cells, the purification of complexes containing DNA ligase IIIα from insect cell lysates, and their characterization by multi-angle light scattering linked to size exclusion chromatography and negative stain electron microscopy.

Keywords

Baculovirus; Bacmid; Insect cells; Affinity chromatography; Size exclusion chromatography; Ion exchange chromatography; Multiple angle light scattering; Negative stain electron microscopy

1 Introduction

It is now generally accepted that many fundamental processes are performed by multi-protein machines. In order to understand how these machines work, it is necessary

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11. Store baculovirus at 4 °C in a cardboard box, or use foil to protect the virus from light. The shelf-life of baculovirus is at least 6 months, up to 1 year. However, the stability of every stock is different. Re-test protein expression in 6-well plates every 6 months, and adjust virus amounts accordingly for protein expression.

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to understand the architecture and functional flexibility of these machines. While X-ray crystallography provides snapshots of structures at atomic resolution, flexible and unstructured regions of proteins are refractory to crystallization and, even if contained within protein crystals, are poorly resolved. Thus, most crystal structures tend to be of stable, relatively small well-folded regions of a protein. With the development of cryo-electron microscopy (EM) combined with improvements in detectors and image analysis, it is now possible to determine high-resolution structures of some large protein complexes with the building of structural models facilitated by docking in X-ray structures corresponding to smaller, well-folded parts of the larger complex [1, 2]. As with X-ray crystallography, flexible regions are not well resolved in cryo-EM. Small angle X-ray scattering (SAXS) provides insights into the shape and conformational flexibility of proteins and protein complexes [3–5]. Similar to cryo-EM, improvement in detectors and data analysis have led to increased use of SAXS to characterize proteins and protein complexes [3–6].

Here we describe the use of Bac-to-Bac Baculovirus Expression systems to efficiently produce separate recombinant baculoviruses encoding DNA ligase IIIα (LigIIIα), XRCC1, or TDP1, each of which contains large unstructured, highly flexible regions. The *LIG3* gene, one of three human genes encoding DNA ligases, encodes mitochondrial and nuclear versions of LigIIIα by alternative translation initiation that are very similar in size after removal of the mitochondrial targeting sequence by proteolysis [7, 8]. In the nucleus, LigIIIα forms a complex with the DNA repair scaffold protein XRCC1 that coordinates the activities of multiple DNA repair enzymes [9, 10]. The nuclear LigIIIα-XRCC1 complex acts as a back-up for DNA ligase I in DNA replication, and there is also functional redundancy with the other nuclear DNA ligases in DNA repair [11–13]. In contrast, LigIIIα is the only DNA ligase in mitochondria where it functions in the replication and repair of the mitochondrial genome in the absence of XRCC1 [11, 12, 14]. The *TDP1* gene also encodes nuclear and mitochondrial versions of this enzyme that interact with LigIIIα and remove 3′ tyrosine residues remaining after degradation of stalled topoisomerase I molecules as well as other 3′ adducts [15, 16]. LigIIIα was co-expressed with XRCC1 or TDP1 as well as with both XRCC1 and TDP1, and, after small-scale experiments to detect complex formation, XRCC1-LigIIIα, LigIIIα-TDP1, and XRCC1-LigIII-α-TDP1 complexes were purified in sufficient amounts for subsequent biochemical and biophysical analysis. Because of the presence of BRCT domains in both LigIIIα and XRCC1 that homo- and heterodimerize and the elongated, asymmetric shape of these proteins [5], it is not possible to accurately estimate the mass and stoichiometry of complexes formed by these proteins by comparing their elution position from a size exclusion column with globular protein standards. Here, we describe the use of multi-angle light scattering coupled to gel filtration (SEC-MALS) in order to estimate the absolute molar mass of LigIIIα complexes independent of their elution volume. Negative stain electron microscopy (NS-EM) is a powerful tool to gain insights into the size and shape of protein complexes with other proteins. Depending on the degree of flexibility and heterogeneity, it is possible to classify 2D projections of single particles into 2D classes with increased signal-to-noise ratio and/or proceed to create a 3D consensus map that can be subsequently used for 3D classification into different conformers. Here, we describe methods that were used to visualize LigIIIα complexes by NS-EM.

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2 Materials

All reagents should be prepared using deionized ultrapure water and using HPLC grade reagents, except that media used for bacterial cultures should be prepared using tap water. Buffers used for cell lysis and protein purification must be vacuum filtered and pre-cooled in 4 °C refrigerator prior to use. Thorough waste disposal and safety regulations must be followed when disposing waste materials.

2.1 Expression of LigIIIα Complexes in Insect Cells

1. pFastBac baculoviral transfer vectors, including pFastBac/LigIIIα, pFastBac/LigIIIα-Strep, pFastBac/6His-XRCC1, pFastBac/Flag-TDP1, and pFastBac/6His-Flag-TDP1 [17, 18], are available from the Expression and Molecular Biology Core directed by Tsai (*see* Note 1).
2. DH10Bac chemical competent cells.
3. Buffers P1, P2, and P3 and TE Buffer for bacmid preparation: Buffer P1: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 100 μg/mL RNase A. Buffer P2: 0.2N NaOH and 1% SDS. Buffer P3: 3 M potassium acetate, pH 5.5. TE Buffer: 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA.
4. CellFECTIN II transfection reagent.
5. Insect cell lines Sf9 (*Spodoptera frugiperda*) and High Five cells (BTI-TN-5B1-4 *Trichoplusia ni*) cultured in ESF 921 protein-free medium (Expression Systems).
6. Cell culture plasticware (6-well and 100-mm tissue culture-treated plates) and reusable, autoclavable polycarbonate non-baffled shaker flasks (125, 250, 500, and 1000 mL).
7. Environmental chamber incubators with orbital shakers maintained at 27 °C.
8. Cell lysis buffer for Sf9 or HF cells: 50 mM NaH_2PO_4, pH 7.5, 300 mM NaCl, 0.5% Igepal, 1 mM PMSF, 10 mM imidazole, and EDTA-free protease inhibitor tablet.
9. Dounce homogenizer.
10. 1× TAE buffer for DNA gel electrophoresis: 40 mM Tris-acetate and 1 mM ETDA, pH 8.3.

[1]. To express multi-protein complexes in insect cells, typically, separate viruses for each protein of interest are produced, and then cells are co-infected with multiple viruses at once. This approach allows flexible combinations of individual viruses to create protein complexes of interest, but often requires extensive optimization of co-infection ratios of these viruses and becomes challenging when more than two viruses are used. Moreover, the probabilities of co-infection of single cells with more than two viruses have been shown to be low [26]. We have developed a multigene baculovirus system, called MacroBac [27], for efficient cloning and assembly of multigenes in the same shuttle vector, thus producing a single baculovirus carrying multiple proteins of interest. Using the MacroBac system significantly increases the reproducibility of protein complex purification in terms of expression levels and subunit stoichiometry. However, the MacroBac single virus does not always work effectively when interactions of proteins in the complex are transient and dynamic, or when there is a vast difference in their sizes. The optimal strategy for efficient production of large, multi-protein complexes should be determined empirically, but can be achieved successfully by co-infection with two MacroBac viruses.

11. 1× Tris-Glycine SDS running buffer for protein gel electrophoresis: 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3.

12. 1× Tris-glycine buffer for Western blot transfer: 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3.

13. Gel electrophoresis apparatus for DNA and proteins and Bio-Rad Mini Trans-Blot transfer apparatus.

14. Ni-NTA Fast Flow resins (Qiagen) and anti-Flag M2 resins (Sigma). Anti-His (Qiagen) and anti-Flag (Sigma)-mouse monoclonal antibodies.

2.2 Purification of LigIIIα Protein Complexes

2.2.1 Buffers

1. Cell lysis buffer: 50 mM sodium phosphate buffer (mono and dibasic) pH 8.0, 300 mM NaCl, 0.5% Igepal, cOmplete mini EDTA free protease inhibitor cocktail.

2. Buffers for ÄKTA FPLC: 40 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM benzamidine, 0.2 mM PMSF (phenylmethylsulfonyl fluoride) with NaCl depending on the buffer P0 (0 M NaCl), P200 (200 mM NaCl), and P1000 (1 M NaCl). P200 + I600 buffer corresponds to P200 buffer containing 600 mM imidazole (*see* Note 15).

2.2.2 ÄKTA FPLC and Columns

1. ÄKTA FPLC (GE Healthcare) with Unicorn software.

2. ÄKTA FPLC columns: HisTrap HP 1 mL (GE Healthcare), HiTrap Q 1 mL (GE Healthcare), HiTrap SP 1 mL (GE Healthcare), dsDNA cellulose from Calf Thymus DNA (Sigma-Aldrich), and HiLoad Superdex 200 16/600 (GE Healthcare) (*see* Notes 18 and 19).

3. Econo column chromatography (Bio-Rad, Cat# 7372511).

2.2.3 Other Equipment

1. Branson Sonifier 150: set to level 6.

2. Beckman Coulter Optima XPN-80 Ultracentrifuge and Ti70 rotor.

3. Bachman Coulter polycarbonate centrifuge bottles, capacity 26.3 mL.

4. VWR Sterile Syringe Filter w/ 0.45 μm cellulose acetate membrane.

5. Millipore Sigma Amicon Ultra 4 (or 15) centrifugal filter unit.

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15. Benzamidine and PMSF should be added to the buffers right before using them in the ÄKTA FPLC.

18. While using a disposable HiTrap FPLC column, make sure the amount of protein loaded onto the column is within the binding capacity of the column beads.

19. Re-useable columns should be stored in 20% ethanol to prevent bacterial growth. Prior to equilibration in running buffer, flush stored columns with water to remove the ethanol.

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2.3 Characterization of LigIIIα Protein Complexes

2.3.1 SEC-MALS

1. Gel filtration running buffer: 40 mM HEPES pH 8, 200 mM NaCl, 10% glycerol, 0.1 mM Tris (2-carboxyethyl) phosphine (TCEP or a similar reducing agent, such as dithiothreitol). The buffer must be compatible with the gel filtration column and with the sample under study. All solutions should be sterile filtered and degassed.
2. Gel filtration column: 10/300 GL Superdex 200 Increase or similar, depending on expected size of protein complex.
3. Fast Protein Liquid Chromatography (FPLC) system, such as ÄKTA pure (GE Healthcare), ÄKTAmicro (GE Healthcare), DuoFlow (Bio-Rad), Alliance (Waters Corporation). Typically, there is an integrated UV detector able to measure absorbance at 280 nm. UV measurement is optional for SEC-MALS, but it facilitates monitoring elution on the FPLC without using the subsequent differential refractometer. For membrane protein analysis, a UV detector is mandatory.
4. MALS and differential refractometer detectors, such as those offered by Wyatt Technology, Malvern Panalytical, and Waters Corporation.
5. SEC-MALS Analysis Software, such as ASTRA (Wyatt Technology). Here, we use ASTRA version 6.1.6.5.
6. Purified protein standard for calibration should be monodisperse under the combination of the chosen column, flow rate, and buffer (typically BSA for the 10/300 GL Superdex 200 Increase column).

2.3.2 NS-EM

1. Glutaraldehyde (GLT).
2. 1 M Tris or Glycine buffered to the pH of the preferred protein solution.
3. Table-top centrifuge.
4. Spin-column concentrators, such as Amicon Ultra 0.5 mL (10 kDa molecular weight cutoff).
5. 12% SDS PAGE gel and running apparatus.
6. Glow discharge system, such as PELCO easiGlow (Ted Pella Inc.).
7. Uranyl formate or uranyl acetate. Uranyl salts are toxic and mildly radioactive; proper precautions must be taken when handling them.
8. Whatman No. 1 filter paper.
9. Transmission electron microscope capable of operating at room temperature, such as Tecnai 12 (FEI).

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3 Methods

3.1 Expression of LigIIIα Complexes in Insect Cells

3.1.1 Transposition

1. Prepare Luria agar plates consisting of 10 g peptone, 5 g yeast extract, 10 g sodium chloride, and 12 g agar in 1 L of tap water. Autoclave and cool down to 55 °C, and add the following to cooled agar solution: 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100–200 μg/mL Bluo-gal, and 40 μg/mL IPTG. Mix the agar solution, and pour 20–25 mL per 100-mm Petri dish under sterile conditions (*see* Notes 2 and 3).
2. Transform 50–100 ng pFastBac plasmid into 100 μL DH10Bac cells using the general transformation protocol. Add 900 μL SOC media to the heat-shocked cell-plasmid mixture, and incubate at 37 °C at 250 rpm for 4 h.
3. Dilute and plate cells on Luria agar plates to obtain 100–200 colonies per plate. Incubate for 18–20 h at 37 °C. Store the agar plates at 4 °C for 8 h on the following day. Then place the agar plates back to 37 °C, and incubate for another 18–24 h. Blue colonies become evident after 48 h post-transformation.

3.1.2 Isolation of Recombinant Bacmid DNA

1. Pick five white colonies from the plate. Inoculate each single white colony into 2 mL LB with tetracycline, gentamycin, and kanamycin, and incubate at 37 °C at 250 rpm for 18–20 h.
2. Spin down 1.5 mL overnight culture in a microtube at 14,000 × *g* for 3 min. Remove the supernatant, and resuspend bacterial pellets in 0.3 mL Buffer P1. Add 0.3 mL Buffer P2, gently mix, and incubate at room temperature for 5 min. Add 0.3 mL Buffer P3, mix gently, and incubate on ice for 5–10 min. Centrifuge at 14,000 × *g* for 10 min (*see* Note 4).
3. Transfer the supernatant to a new tube containing 0.65 mL isopropanol. Mix by inverting the tube a few times, and incubate on ice for 10 min. Centrifuge the sample at 14,000 × *g* for 15 min at room temperature. Pour to discard the supernatant. Add 0.5 mL 70% ethanol to each tube, and invert the tubes a few times to wash the DNA pellet. Centrifuge for 10 min at 14,000 × *g* at room temperature. Carefully remove the supernatant as much as possible using a micropipette. Do not pour to discard ethanol as the DNA pellet may be dislodged. Air dry the bacmid DNA for 10 min until the pellet becomes transparent, and redissolve in 40 μL TE buffer for at least 15 min. Gently tap the tube to mix, and avoid pipetting up and down (*see* Notes 5 and 6).

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[2]. Good quality of bacmid DNA is key to producing high-titer baculovirus stocks. Take great care in all steps in producing bacmid DNA.

[3]. It is important to prepare Luria agar plates as described. Do not use commercial Luria-Bertani agar that contains tryptone, which does not work for the blue/white selection using Bluo-gal.

[4]. Purify bacmid DNA from 1.5 mL of overnight culture using isopropanol precipitation that generates sufficient amount of DNA for up to 6 transfections. It is not necessary to purify from larger cultures using spin columns as this tends to produce poor quality bacmid DNA.

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4. Restreak the overnight culture on Luria agar plates to verify the white phenotype (*see* Note 7).

5. Analyze 5 μL of bacmid DNA on a 0.5% agarose/TAE gel at 20 V for 18–20 h. Select three bacmid clones that show high intensity of an intact signature bacmid band (Fig. 1) with confirmed white phenotype for transfection (*see* Note 8).

3.1.3 Transfection of Sf9 Insect Cells with Recombinant Bacmid DNA

1. Seed 2 mL of Sf9 cells in 1% FBS media at 0.5 million cells/mL in 6-well plates the day before, and incubate at 27 °C for 18–20 h (*see* Note 9).

2. For each bacmid clone, prepare a transfection mixture as following:

   Tube A: dilute 5–10 μL of bacmid DNA in 100 μL of ESF 921 SFM.

   Tube B: dilute 5 μL of CellFECTIN in 100 μL of ESF 921 SFM.

   Mix Tubes A and B, and incubate at room temperature for 30–45 min.

3. Include one tube without bacmid DNA as a negative control.

4. Wash Sf9 cells twice with 2 mL of ESF 921 SFM.

5. Add 0.8 mL ESF 291 SFM to the transfection mixture (total 1 mL), and mix gently. Aspirate the media from cells, and carefully add 1 mL diluted transfection mixture to each well without disturbing the attached cells. Also include one well without adding transfection mixture as a control. Then incubate at 27 °C for 5 h.

6. Aspirate transfection mixture, add 2 mL fresh 1% FBS media per well, and incubate at 27 °C for 72–96 h.

7. Harvest virus by transferring the media from each well to a 15-mL conical tube. Clarify by centrifugation at 500 × *g* for 5 min, and transfer the supernatant to a new 15-mL conical tube. This is designated as P0 clones. Store at 4 °C protected from light.

3.1.4 Baculovirus Amplification and Screening

1. Plate 10 mL of Sf9 cells in 1% FBS media at one million cells/mL in 100-mm Petri dishes the day before. Plate four plates for three clones and including an uninfected control. Add 250 μL of each P0 clone to Sf9 cells, gently swirl the plate to mix, and incubate at 27 °C for 72 h.

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[5]. Use clear microcentrifuge tubes when precipitating bacmid DNA by isopropanol, as it should be a small DNA pellet of 1–1.5 mm in diameter. Avoid overprecipitation during bacmid preparation. Discard bacmid samples, and start over if a large, white precipitate (>2 mm in diameter) is obtained.

[6]. Do not overdry bacmid DNA. Add TE buffer as soon as bacmid DNA becomes transparent, and allow DNA to dissolve for a minimum of 15 min. Gently tap the tubes to dissolve. Avoid pipetting up and down bacmid DNA.

[7]. Always restreak the white colonies to ensure the white phenotype of the bacmid clones before transfection.

[8]. Always analyze bacmid DNA by agarose gel electrophoresis to ensure intact, unbroken bacmid is present (Fig. 1) before use for transfection.

[9]. We grow Sf9 cells in media supplemented with 1% fetal bovine serum (FBS) to produce baculovirus stocks for better viral stability, and use Sf9 cells maintained in serum-free media for protein expression. Both Sf9 and High Five cells are maintained in antibiotic-free media. Antibiotics are added at half-strength (0.5× concentration) in large-scale cultures for protein expression.

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2. Transfer the media to a 15-mL conical tube, centrifuge at 500 × *g* for 5 min, and transfer the supernatant to a new 15-mL conical tube. This is designated as P1 clones. Store virus stocks at 4 °C and protected from light.

3. Plate 10 mL of Sf9 cells in 1% FBS media at 1 million cells/mL in 100-mm Petri dishes the day before. Add 100 μL of P1 clones to Sf9 cells, gently swirl the plate to mix, and incubate at 27 °C for 72 h.

4. Transfer the media to a 15-mL conical tube, centrifuge at 500 × *g* for 5 min, and transfer the supernatant to a new 15-mL conical tube. This is designated as P2 clones. Store virus stocks at 4 °C and protected from light.

5. Seed 2 mL of High Five cells at 0.5 million cells/mL in 6-well plates, and allow cells to attach for 30 min. Infect High Five cells with 20 μL of P2 clones, gently swirl the plate to mix, and incubate at 27 °C for 45–48 h.

6. Resuspend High Five cells directly in the media, transfer cells to 2 mL microtubes, and centrifuge at 500 × *g* for 5 min to pellet the cells. Aspirate the media, resuspend cell pellets in 150–200 μL 1× SDS loading buffer, and heat denature at 95 °C for 5 min.

7. Total protein expression is analyzed by resolving protein samples (8–10 μL loading per lane) on SDS-PAGE and analyzed with Coomassie staining and Western blotting using antipenta-His antibody to detect 6×His-tagged XRCC1 and anti-Flag antibody to detect Flag-tagged TDP1. Select the clone with the best expression for each protein to scale up virus production.

3.1.5 Scaled-Up Baculovirus Production in Suspension Cultures

1. Plate 100 mL of Sf9 cells in 1% FBS media at 1 million cells/mL in a 250-mL shaker flask the day before.

2. To produce P2 stocks, add 1 mL of selected P1 clone to 100 mL cells. Gently swirl the flask to mix and allow cells to sit at 27 °C for 1 h. Then, transfer the flask to an orbital shaker, and incubate at 140 rpm for 72h at 27 °C.

3. Transfer the media to 50 mL conical tubes, centrifuge at 500 × *g* for 5 min to pellet any floating cells, and transfer the supernatant to new 50-mL conical tubes. This is designated as P2ii stocks. Store virus stocks at 4 °C and protected from light (*see* Notes 10–12).

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[10]. Using the described procedures above, high-titered P2ii stocks in the range of 2–10 × 10^8 pfu/mL (i.e., plaque-forming unit per mL of virus) can be reproducibly generated. Traditionally, the viral titers or the infection potency of a viral stock may be determined by plaque formation in immobilized monolayer culture, which is a tedious and long procedure, taking up to 10 days to complete. However, optimal infection conditions vary. The viral titers do not always correlate directly to protein expression levels. The optimal MOI (multiplicity of infection, aka viral particles per cell) has to be determined empirically for each protein of interest. We use a proxy titration method to determine the optimal MOI for protein expression by infecting insect cells with varying volumes of virus, from as low as 1 μL, up to 40 μL, per million cells, and select the best virus/cell ratio based on the total protein expression analysis. This method saves time and streamlines the expression workflow with consistent expression for protein of interest.

[12]. For long-term storage, add 10% FBS to the baculovirus stock, that is, mix 0.5 mL FBS with 4.5 mL virus, and store at −80 °C in 1 mL aliquots. After thawing, one vial of virus can infect up to 100 mL of fresh Sf9 cells, and produce 100 mL new viral stock. Always perform expression tests in six-well plates or small-scale shaker cultures to confirm expression and/or protein complex formation before scaling up expression.

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4. To test protein expression by the new viral stocks, seed 2 mL of High Five cells as described in **step 5** ***from*** Subheading 3.1.4. Infect High Five cells with 5–40 μL of P2ii stocks per well for 45–48 h at 27 °C. Collect infected cells, and analyze protein samples as described in **steps 6** and **7** ***from*** Subheading 3.1.4.

3.1.6 Co-expression of LigIIIα, XRCC1, and TDP1 in Suspension Insect Cells by Co-infection with Two or Three Baculoviruses

1. To test single protein expression of LigIIIα, XRCC1, and TDP1 in shaker cultures, plate 30 mL of High Five and 50 mL of Sf9 cells at 1 million cells/mL in 125-mL shaker flasks. Add P2ii stocks to insect cells with the amount of 5–40 μL virus per million cells. Gently swirl the flask to mix, allow to sit still for 1 h, and then incubate at 140 rpm for 45–48 h for High-five cells and for 68–72 h for Sf9 cells. Cells (1 mL) were sampled and analyzed for total protein expression by SDS-PAGE and Western blotting as described above. The rest of the cells are pelleted and analyzed for solubility and for affinity purification where applicable (*see* Notes 13 and 14).

2. Resuspend High-five (30 mL) or Sf9 (50 mL) cells in 5 mL of lysis buffer, and lyse cells by Dounce homogenizer using a type B, or tight-fitting pestle with repeated 40 strokes. Clarify cell lysates by centrifugation at 15,000 × *g* for 30 min at 4 °C. Sample 50 μL of cell lysates before (total protein fraction) and after (soluble protein fraction) clarification, and mix with 50 μL of 2× SDS loading buffer. Analyze protein solubility by comparing total vs. soluble fractions using SDS-PAGE and Western blotting.

3. Based on the initial shaker expression analysis of each protein in **steps 1** and **2**, plate insect cells as described in **step 1**, co-infect High Five and Sf9 cells with the virus stocks of various ratios, incubate on a shaker for 48–72 h depending on cell lines, and then collect cells for total protein analysis and solubility analysis as described in **step 2**.

3.1.7 Affinity Co-purification of LigIII Protein Complexes

1. Lyse co-infected insect cells as described in **step 2** ***in*** Subheading 3.1.6.

2. Remove the storage buffer from the selected affinity resins, for example, Ni-NTA beads, and then equilibrate the resins with the same lysis buffer in 1.5 mL microtubes. Use a column volume of 50 μL for 30–50 mL insect cells. Transfer the resins to a 15 mL conical tube.

3. Add soluble extracts to the resins, and allow for batch binding at 4 ° C with gentle rocking for 1 h. Centrifuge the tube at 500–700 × *g* for 5 min to collect the

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[13]. When expressing protein complexes using more than two viruses to infect insect cells, test combinations of viral ratios in small-scale shaker cultures, and perform an affinity co-purification to select the best co-infection ratio that yields a protein complex of the best stoichiometry (Fig. 2). To test viral ratios, keep the virus amount constant for the difficult-to-express or larger-sized proteins, and adjust (decrease) the virus amount for the easier-to-express or smaller-sized proteins.

[14]. Expression conditions determined from 30 to 50 mL cultures are often scalable to 400-mL or larger cultures. However, it is best to run a scale test in the desired culture volume using two of the best possible ratios for a time course and establish optimal expression conditions before scaling up.

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resins. Transfer the supernatant (aka flow through fraction) to a new tube. Wash the resin 3 times with 10 mL of Wash Buffer (same as the Lysis Buffer with increased imidazole to 20–25 mM), then transfer resins to a 1.5 mL microtube, and wash 4 times with 1 mL of wash buffer. Centrifuge the tube at 500–700 × *g* for 5 min to collect the resins. Elute proteins from the affinity resins by adding 1 column volume of elution buffer (same as lysis buffer with increased imidazole to 300 mM) and incubating on ice for 5 min, followed by centrifugation. Transfer the supernatant (aka eluted fractions) to new 1.5 mL microtubes. Elute two times. For Flag purification with anti-Flag M2 resins, binding and washing buffers are the same as lysis buffer, and elute proteins from the resins using lysis buffer supplemented with 200 μg/mL 3× Flag peptide and incubate on ice for 15 min.

4. Sample purification fractions (including column load, flow through, eluted fractions, and leftover resins), mix with an equal volume of 2× SDS loading buffer, and heat denature at 95 °C for 5 min. Analyze purification fractions by SDS-PAGE against BSA standards with Coomassie staining and/or Western blotting (Fig. 2).

3.1.8 Scaled-Up Co-expression in Insect Cells

1. After determining the best co-infection ratio and cell line for LigIIIα, XRCC1, and TDP1 from small-scale co-expression and co-purification, plate 400 mL of insect cells at 1 million cells/mL in 1000-mL shaker flasks. Infect cells with the virus amounts, and perform a time course test, that is, sampling cells at 40, 44, and 48 h post-infection for High Five cells and 48, 60, and 72 h for Sf9 cells. Analyze total protein expression by SDS-PAGE and Western blotting, or perform a small-scale affinity co-purification from sampled 25 mL coinfected cells to confirm LigIIIα complexes.

2. Scale up expression of LigIIIα complexes using the finalized optimal conditions, including selected cell line, viral co-infection ratio, and infection time.

3.2 Purification of LigIIIα Protein Complexes

LigIIIα/6His-XRCC1, LigIIIα/6His-Flag-TDP1, and LigIIIα/6His-XRCC1/Flag-TDP1 protein complexes are purified using the chromatography columns in the order shown in Table 1.

3.2.1 Lysate Preparation

1. Add 20 mL of cell lysis buffer and two cOmplete mini EDTA free protease inhibitor cocktail tablets per 0.4 L cell pellet, and thaw on ice (*see* Note 15).

2. Sonicate 25 s, and rest on ice for 30 s. Repeat this four more times (*see* Note 16).

3. Transfer the sonicated lysate in the Beckman Coulter polycarbonate centrifuge bottles, and balance the weight for centrifugation.

[16] Keeping lysates on ice in between sonication cycles is crucial in limiting proteolysis and degradation.

4. Centrifuge the lysates at 26,600 × *g*, at 4 °C, for 30 min.

5. Take the clear supernatant, and filter using a sterile syringe filter with 0.45 μm cellulose acetate membrane. Keep the lysate tube on ice throughout the filtering process (*see* Note 17).

3.2.2 Nickel Column FPLC Using HisTrap HP Column

1. For this purification step P200 is used as buffer A and P200 + I600 as buffer B. Following the manufacturer's guideline, equilibrate the HisTrap HP (1 mL) disposable column with 5% buffer B (P200 + I600 buffer) (*see* Notes 18 and 19).

2. Add imidazole (1 M stock solution) to the filtered lysate right to a final concentration of 30 mM before loading the sample lysate into the sample loading loop for the ÄKTA FPLC system (*see* Note 20).

3. Load the sample to the column selecting 5% buffer B and with a flow rate of 0.5 mL/min. Follow the manufacturer's guideline for setting limit for high back pressure alarm.

4. Start collecting flow through as soon as the UV absorbance (280 nm) peak starts going up, and collect till the peak drops near to the basal UV absorbance (280 nm).

5. Stop sample loading, and continue to wash the column with 5% buffer B till the UV reading (280 nm) becomes a flat line.

6. Elute the His-tagged protein bound to the HisTrap HP column in a stepwise manner using 8.3% (50 mM imidazole), 16.7% (100 mM imidazole), 50% (300 mM imidazole), and 100% (600 mM imidazole) buffer B. The elution fraction size should be 1 mL for each step, collecting 10 elution fractions for each step (*see* Note 21).

7. Based on the UV absorbance peak (280 nm), select elution fractions, and run an SDS-PAGE gel to verify and identify fraction containing the protein complex of interest. Upon verification, save the fractions containing the protein complex of interest for the next protein purification step (Fig. 3) (*see* Note 24).

3.2.3 Size Exclusion Column FPLC Using HiLoad Superdex 200 16/60

1. Prior to running a sample, equilibrate the HiLoad Superdex 200 16/60 column with two column volumes (120 mL × 2) of P200 buffer in ÄKTA FPLC (*see* Notes 19 and 22).

[17]. Filtering the cell lysate after the centrifugation step helps to remove residual debris and particulates, which, if not removed, can cause high back pressure during the FPLC runs.
[20]. Adding 30 mM imidazole to the lysate before loading on the nickel column helps in reducing non-specific protein binding.
[21]. For stepwise elution, wait until the UV absorbance becomes flat before moving on to the next step. This usually occurs within ten fractions for the nickel column but if not, wait until it becomes flat and then move on to the next step.
[24]. It is important to process the eluted fractions for the next purification step or store (at −80 °C freezer) as soon as they come out of the ÄKTA system. This will limit protein complex degradation.
[22]. Before running the size exclusion column step, it is important to calibrate the column with protein standards. This determines the void volume and expected elution positions of proteins (volume) according to their molecular weight.

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2. Load samples from 0.5 to 5 mL onto Superdex 200 16/60 column. If nickel column fraction sample volume is greater than 5 mL, then concentrate the sample in P200 buffer using an Amicon Ultra 4 (or 15) centrifugal filter unit (*see* Notes 23 and 26).
3. Load the sample at 0.3–0.5 mL/min, and elute with P200 buffer. It is important to make sure no air bubble gets inside the column while sample is loading. Follow the manufacturer's guideline for setting limit for high back pressure alarm.
4. Start collecting 2 mL elution fractions after the void volume has passed, and continue collection for total 40 tubes.
5. Based on the UV absorbance peak (280 nm), select elution fractions, and run an SDS-PAGE gel to verify and identify fraction containing the protein complex of interest. Upon verification, pool the fractions containing the protein complex for the next protein purification step, or store them in −80 °C freezer (*see* Note 24).

3.2.4 dsDNA Cellulose Column FPLC

1. Pack a disposable econo column using 0.7–1 g of dsDNA cellulose from Calf Thymus DNA. The column bed length and diameter should be around 1 and 0.75 in., respectively. To equilibrate the column, use P100 buffer, which can be made by diluting P200 buffer twofold with P0 buffer. Equilibrate the column with 3× 10 mL P100 buffer using gravity (*see* Note 25).
2. For this purification step, P0 and P1000 buffers are used as buffers A and B.
3. Before loading the sample, dilute the sample so that the final NaCl concentration is 100 mM. Load the sample to the column selecting 10% buffer B (100 mM NaCl) and with a flow rate of 0.5 mL/min. Set the alarm for high backpressure to 0.5 mPa.
4. Start collecting flow through as soon as the UV absorbance peak (280 nm) starts going up, and collect till the peak drops near to the basal UV reading (280 nm).
5. Stop sample loading, and continue to wash the column with 10% buffer B until the UV absorbance (280 nm) reading becomes a flat line.
6. Elute the protein complex bound to the dsDNA cellulose column using a linear gradient by selecting buffer B range from 10 to 80% and length for 40 min. Collect 0.5 mL fractions and a total of 40 elution fractions.

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23. While loading the sample in a size exclusion column, it is very important not to introduce air bubbles into the column. To avoid bubbles, add buffer to the top of the sample loading loop inlet, and then connect the injection port tube on that end while keeping the bottom outlet of the sample loading loop open and unattached to the other injection port tube. As soon as samples start to come out of the bottom end as droplets, connect the other injection port tube. This will stop air bubbles from getting into the column through the sample loading process.

26. For concentrating, eluted protein complexes with Amicon Ultra 4 or 15 centrifugal filter units. The cutoff value for the filter molecular weight should be selected to be smaller than the protein complex molecular weight. These units can also be used for buffer exchange.

25. While packing the disposable econo column for dsDNA cellulose from calf thymus, wash the dsDNA cellulose via centrifugation in falcon tubes before packing them into the column using gravity.

7. Based on the UV absorbance peak (280 nm), select elution fractions, and run an SDS-PAGE gel to verify and identify fraction containing the protein complex of interest. Upon verification, save the fractions containing the protein complex for the next protein purification step, or store them in −80 °C freezer (Fig. 4) (*see* Note 27 and 28).

3.2.5 Ion-Exchange Column FPLC (HiTrap Q or HiTrap SP)

1. Use P0 and P1000 as buffers A and B for both the HiTrap Q and SP columns (*see* Note 18).
2. Equilibrate the column with 5 mL of 10% buffer B, and dilute the sample fractions so that the final NaCl concentration is 100 mM.
3. Follow **steps 3–7** from the Subheading 3.2.4 *dsDNA cellulose Column FPLC* (Fig. 5) (*see* Note 27 and 28).

SEC-MALS analysis can be carried out at room temperature or in a refrigerated chamber. Some proteins are sensitive to room temperature. If using a refrigerated chamber, be sure to cool down the buffer before use. Note that flow rates must be decreased at lower temperatures to respect the pressure limits of the FPLC system and gel filtration column.

3.3 Characterization of LigIIIα Protein Complexes

3.3.1 Characterization by SEC-MALS

Column and System Equilibration in Running Buffer: A FPLC system is used to equilibrate the gel filtration column with gel filtration running buffer. The chosen buffer must be determined somewhat empirically. An appropriate buffer should not induce precipitation of the sample within a short timeframe. Generally, the optimal buffer has already been determined at the protein purification stage. The result from SEC-MALS may indicate that a buffer leads to sample aggregation but without obvious precipitation. In this case the buffer should be altered by modification of the pH, the buffering molecule, addition or removal of salt, glycerol, reducing agents, detergents, or known ligands.

It is important that the only difference between the sample and the buffer is the protein complex to be measured, as the refractive index of the buffer component will be subtracted from the sample to give the concentration of the sample. Detailed articles on theoretical aspects of SEC-MALS are available elsewhere and will not be discussed here [19, 20].

1. Equilibrate the gel filtration column and detectors with the buffer using the FPLC pumps. Set the appropriate high-pressure alarms, and adjust the flow rate accordingly. Maintain the flow rate to no more than ~90% of the high-pressure

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[27] All the LigIIIα protein complexes were concentrated and stored in P200 buffer (***Buffer***, Subheading 2.2.1) in small aliquots to avoid repeated freeze/thaw cycles that may cause protein degradation or loss of activity. Aliquots should be flash frozen in liquid nitrogen and then transferred to the −80 °C freezer.

[28] Biophysical analysis of protein requires high purity (>95%) and concentration (2–10 mg/mL) of the sample. Concentrations of the purified protein complexes are estimated using the Bradford assay and staining with Coomassie blue after an SDS-PAGE gel. BSA is used as the standard in both assays. The SDS-PAGE gel provides information about the purity of the protein complex with low concentrations revealing the presence of multiple bands with a similar electrophoretic mobility and higher concentrations revealing minor background contaminants.

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limit, as there may be short-lived spikes in the pressure that could trigger the alarm and pause the run. *See* Subheading 4, Note 29.

2. The flow-path should include the gel filtration column, UV detector (optional, typically first detector), MALS flow cell, differential refractometer (last detector), fraction collector (optional).
3. Set the differential refractometer to purge mode to equilibrate both the reference and sample cells.
4. If present, turn on the sonicator in the MALS flow cell (COMET in Dawn HELEOS II from Wyatt Technology) at least 15 min prior to sample injection.
5. When equilibration is finished, turn off purge on the differential refractometer.

Calibration Using a Known Protein and Obtaining the Molar Mass of a Sample: The SEC-MALS system must be calibrated with a monodisperse protein of known size. BSA is typically used if the gel filtration column can efficiently separate the BSA monomer peak from the dimer peak. For example, the BSA peaks overlap in Fig. 6a when injected onto a 3.2/30 Superdex 200 Increase (2.4 mL) column but show clear separation in Fig. 6b when using a 30/300 Superdex 200 Increase (24 mL) column. Good peak separation is important for calibration since the band broadening (**step 7** below) requires clear peak shapes for correction of peak dispersion (Fig. 6c, d). It is necessary to calibrate the SEC-MALS system before each set of experiments, as small changes in the buffer can affect results.

Whereas a monodisperse peak is necessary, it does not necessarily have to come from a monomer. The Aldolase tetramer (157 kDa) has been used successfully in calibrating SEC-MALS systems [21]. Regardless of the calibration standard, its Stokes radius must be known (3 nm for BSA monomer, 4.8 nm for aldolase). The calibration standard must be injected in sufficient quantities to provide strong signals in the MALS detectors. We recommend injecting 500 μg of BSA diluted in gel filtration buffer.

It has been observed that changes to the FPLC pump flow rate result in column shedding of fine particles that are visible by MALS detectors and may affect the baseline. It is recommended to inject samples without changing the pumps. Here we provide an overview of the steps to be taken; exact operation depends on the researcher's specific SEC-MALS system and software.

1. To avoid air entering the FPLC system and the column and to equilibrate the injection loop, change the injection position to *Inject* (or equivalent) to flow running buffer through the injection loop. At this position samples cannot enter the injection loop.
2. Spin down the sample in a table-top centrifuge at top speed for 5 min. Take up the supernatant with an injection syringe, and insert it into the injection valve of the FPLC.

29. Allow equilibration of at least two column volumes. Ensure that the signals from the various detectors are flat. The pressure over the column may have changed now that the column is equilibrated in the buffer. If this is the case, adjust the flow rate accordingly.

3. Change the injection position to *Load* (or equivalent), and push the injection syringe plunger to expel the sample into the injection loop.
4. On the SEC-MALS software (ASTRA), open a New Experiment from Default. When prompted to inject the sample, press *OK*, and quickly change the injection position on the FPLC to *Inject*. The running buffer is now pushing sample through the injection loop onto the column.
5. Once the run has finished, press *Stop* in the ASTRA software, and save the result. To avoid light scattering noise, the flow rate should not be modified between runs, if possible.
6. In the Procedures sections, select the level of despiking, and define the Baselines of the MALS and refractometer detectors (the UV detector is optional). The baseline limits should extend well beyond the peak(s) of interest. Under the Peaks section, define the monodisperse peak (monomer for BSA) by setting the limits to half the maximum peak height. *See* Subheading 4, (*see* Note 30).
7. To calibrate the SEC-MALS, right-click on Configuration, and choose *Alignment*. Highlight the peaks of the displayed detector traces, and click *Align Signals*, and then *Apply*. Next, choose *Brand Broadening* from the Configuration right-click menu, and press *Perform Fit*, using the refractometer as the reference instrument. Finally, right-click on Configuration, and choose *Normalize* and Peak 1 (defined in **step 6**). Enter the radius for the calibration standard (3 nm for BSA monomer), and press *Normalize*.
8. In the Procedures section, choose Molar Mass & Radius from LS. Clicking throughout the BSA monomer peak will show the calculated molar mass at each elution position. *See* Subheading 4, (*see* Note 30).
9. Under Results, the Mw should closely match the known molar mass of the calibration protein.
10. Save the Experiment as Method.

    It is advisable to run the MALS flow-cell sonicator (COMET) between injections. The sample injection should follow the above steps, with the following modifications to steps.

4. In the ASTRA software, choose New Experiment from Method, and select the calibrated method that was saved using a known protein standard.
6. Under the Peaks section, define each peak of interest by setting the limits to half the maximum peak height. If a peak is asymmetric, one can extend the peak limit to estimate the molar mass of the species causing asymmetry.
7. Calibration is *not* performed on samples of unknown size.
8. The peak limits may be adjusted in the Molar Mass & Radius from LS section.

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[30] Any light scattering detectors that have a low peak signal compared to the baseline noise can be disabled in Molar Mass & Radius from LS.

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9. The Mw of each peak and its fraction is presented in the Results Report.

3.3.2 Characterization by Negative Stain Electron Microscopy—
Buffer components that stabilize a protein or protein complex may react adversely with the negative stain used in NS-EM. Therefore, it may be necessary to remove these components prior to preparing a sample for observation. One way to retain the sample's integrity and render it compatible with the stain is to mildly crosslink it [22] and then to exchange the buffer to a buffer suitable for negative staining. Another advantage of crosslinking is to preserve the concentration-dependent interactions that may be lost upon sample dilution. Common crosslinking agents include BS3, DSS, and GLT. Here we describe crosslinking with GLT. It is advisable to first perform a titration series of crosslinker concentrations to find an optimal concentration that leads to the generation of a new high-molecular weight band but minimizes aggregated protein in the well of the SDS-PAGE gel. We have found that 0.05% (v/v) GLT works well for LigIIIα complexes. Examples of successful crosslinking of LigIIIα complexes are shown in Fig. 7a.

Mild Chemical Crosslinking of DNA Ligase III Samples

1. Prepare a 10× solution of GLT in the same buffer as the protein complex, and protect from light with aluminum foil.
2. With the protein sample on ice, add 10× GLT to a final concentration of 1×. *See* Subheading 4, (*see* Note 31).
3. Leave for 5 min on ice in the dark.
4. Add 1 M buffered Tris or Glycine to final concentration of 50–100 mM final to quench unreacted GLT.
5. Pipette the sample into a spin column, and dilute with NS-EM buffer. *See* Subheading 4, (*see* Note 32).
6. Centrifuge for 5 min.
7. Dilute the spin column retentate further with NS-EM buffer.
8. Repeat **steps 6** and **7** until a sufficient buffer exchange has taken place (typically three 50-fold dilutions).
9. Load samples of non-crosslinked and crosslinked proteins onto an SDS-PAGE gel, stain, and evaluate the crosslinking efficiency (*see* Fig. 7a).

Negative Stain Electron Microscopy: Detailed protocols and variations on the NS-EM technique are well documented elsewhere [23–25]. Here we present a simple overview of NS-EM grid preparation of LigIIIα complexes. A grid with a moderate particle concentration is shown in Fig. 7b. Single particle image analysis is not discussed, as different software have their own workflows.

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31. The crosslinking is typically carried out in small volumes (20 μL).
32. In order to recover the original small volume sample without excessive dilution, it is recommended to use a 0.5 mL spin column, as the dead volume is typically 50–60 μL.

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1. Prepare a solution of uranyl acetate (1–2%) or uranyl formate (1–1.5%). Uranyl acetate may be stored for 1 year at 4 °C, whereas uranyl formate can only be stored for 1–2 days at room temperature. *See* Subheading 4, (*see* Note 33).
2. Glow-discharge carbon-coated copper grids for 30 s with 15 mA plasma current, or similar, with the carbon side exposed to the air.
3. With a grid clamped in negative action tweezers, carefully pipette 5 μL of sample onto the grid, and let incubate 1 min. *See* Subheading 4, (*see* Note 34).
4. Blot excess sample solution using Whatman paper.
5. Immediately pipette 5 μL of negative stain, and let incubate 1 min.
6. Blot off the negative stain using Whatman paper.
7. Remove the grid by advancing Whatman paper between the tweezer arms.
8. Allow the grid to air dry for 5 min.
9. Insert the grid into a transmission electron microscope, and evaluate the particle concentration. *See* Subheading 4, (*see* Note 34) if the concentration is not ideal.
10. Collect micrographs and carry out single particle analysis.

Acknowledgments

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[33]. Uranyl formate requires an adjustment of pH after dissolving in boiling water. Typically 2 μL of 5 M NaOH is added to 500 μL of solution and allowed to equilibrate, and then the solution is centrifuged to remove precipitated uranyl formate.

[34]. We have found that a sample concentration of ~25–50 nM incubated for ~1 min results in a moderate concentration of particles in the field of view. If the sample concentration is unknown, one may use serial dilutions to find an optimal particle concentration. If the undiluted sample results in too few particles, one may increase on-grid incubation time or apply the sample multiple times after blotting excess.

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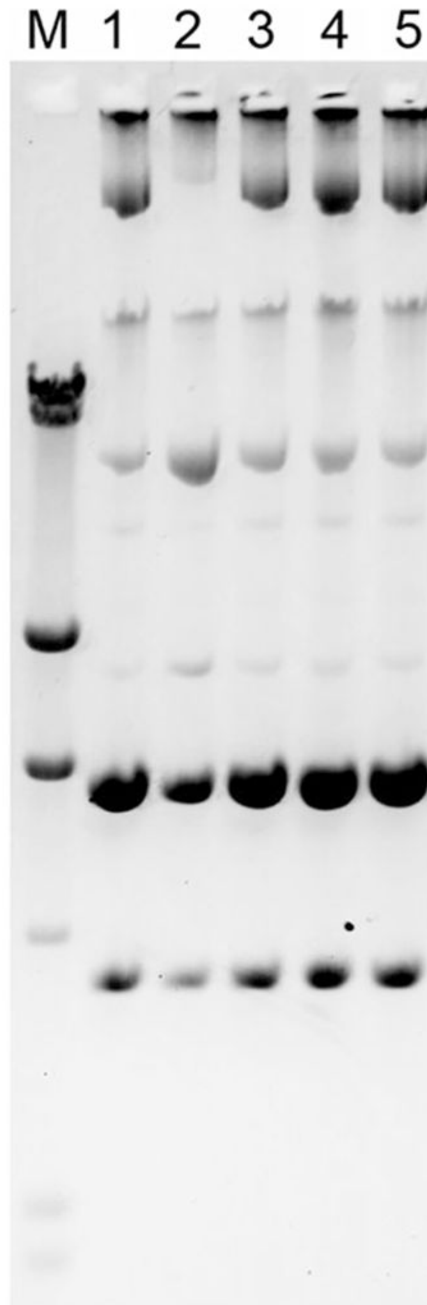

Fig. 1.

Analysis of recombinant bacmid DNA by 0.5% agarose gel electrophoresis at 20 V for 18–20 h. Bacmid DNA is isolated from five clones per construct (lanes 1–5). Lambda DNA HindIII digest containing 23.1, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 kb fragments is used as a DNA size marker (lane M). The signature intact bacmid DNA band is right above the 23.1 kb fragment as indicated by an arrow

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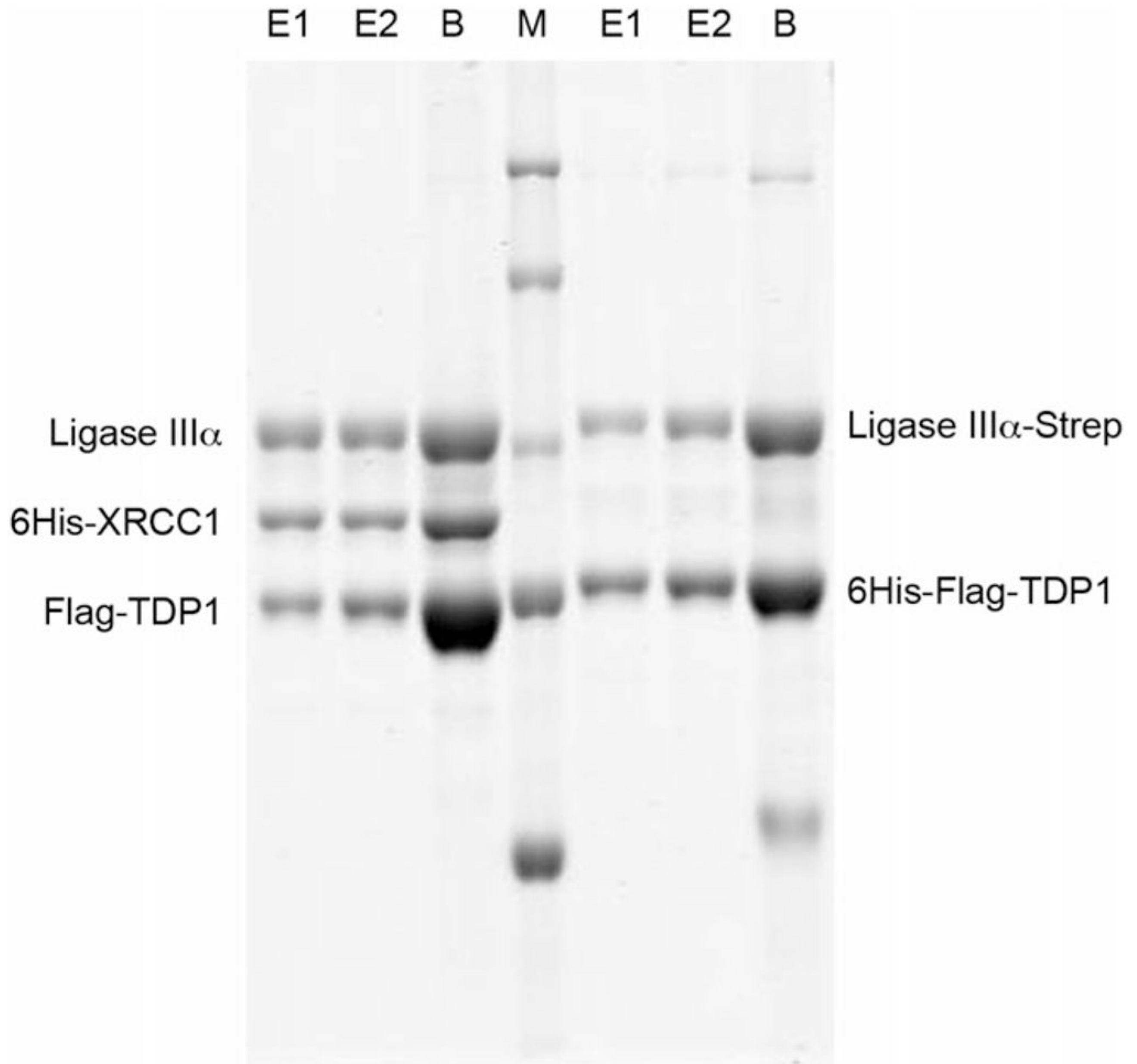

Fig. 2.

Small-scale affinity purification of LigIII-XRCC1-TDP1 trimeric complex (left side of gel) and LigIII-TDP1 dimeric complex (right side of gel) from 25 mL of co-infected Sf9 cells using Ni-NTA and anti-Flag M2 columns, respectively. Approximately 10% of first (E1) and second (E2) eluates and 20% of leftover beads (B) were analyzed by 7.5% Tris-Glycine SDS-PAGE with Coomassie staining. Protein molecular weight standards (M) from the top of the gel are 250, 150, 100, 75, and 50 kDa

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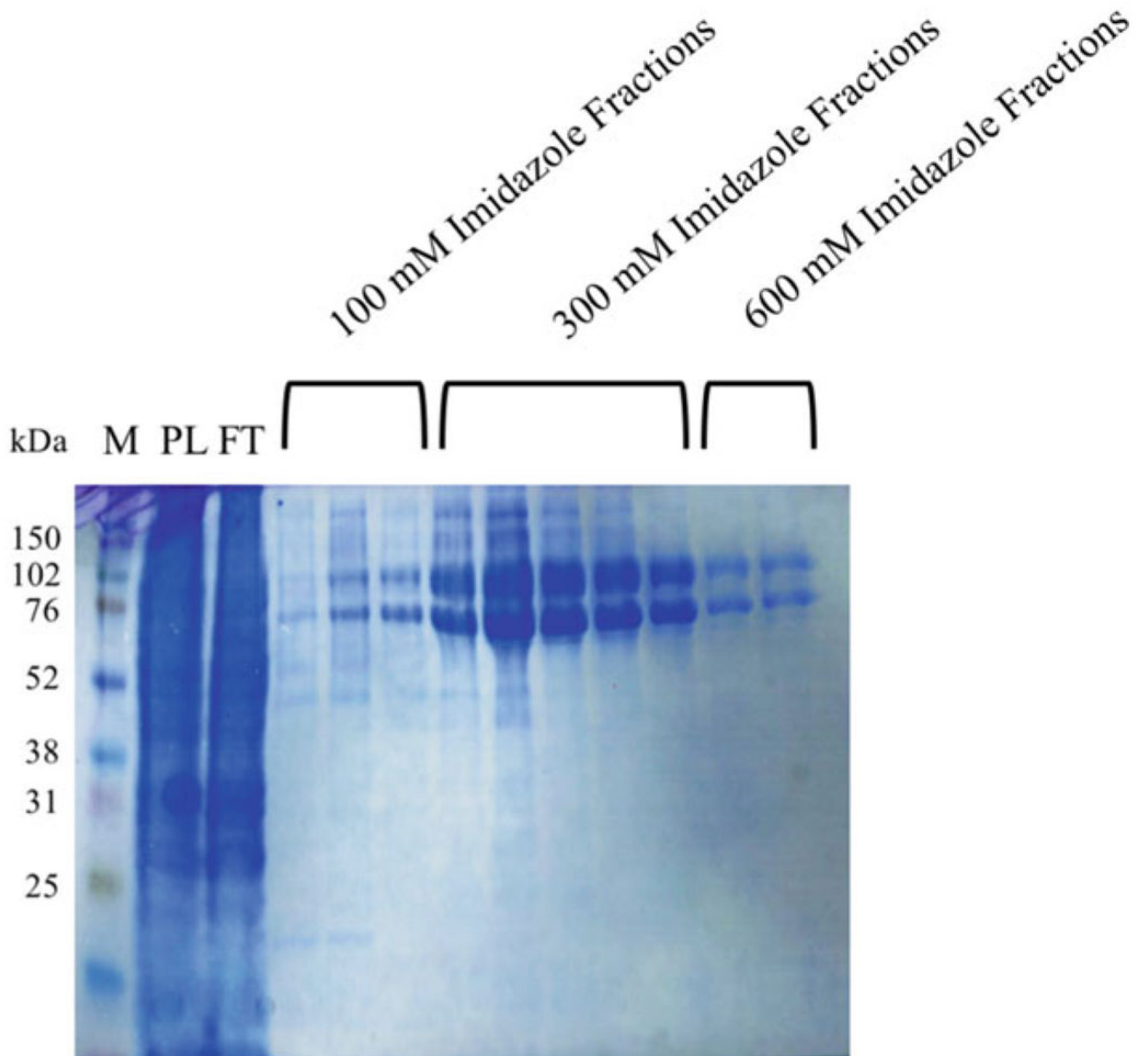

Fig. 3.

Purification of LigIIIα/TDP1 complex using a nickel column. PL and FT represent pre-load and flow through, respectively. Proteins in 100, 300, and 600 mM imidazole eluates were detected by Coomassie staining after separation by SDS-PAGE

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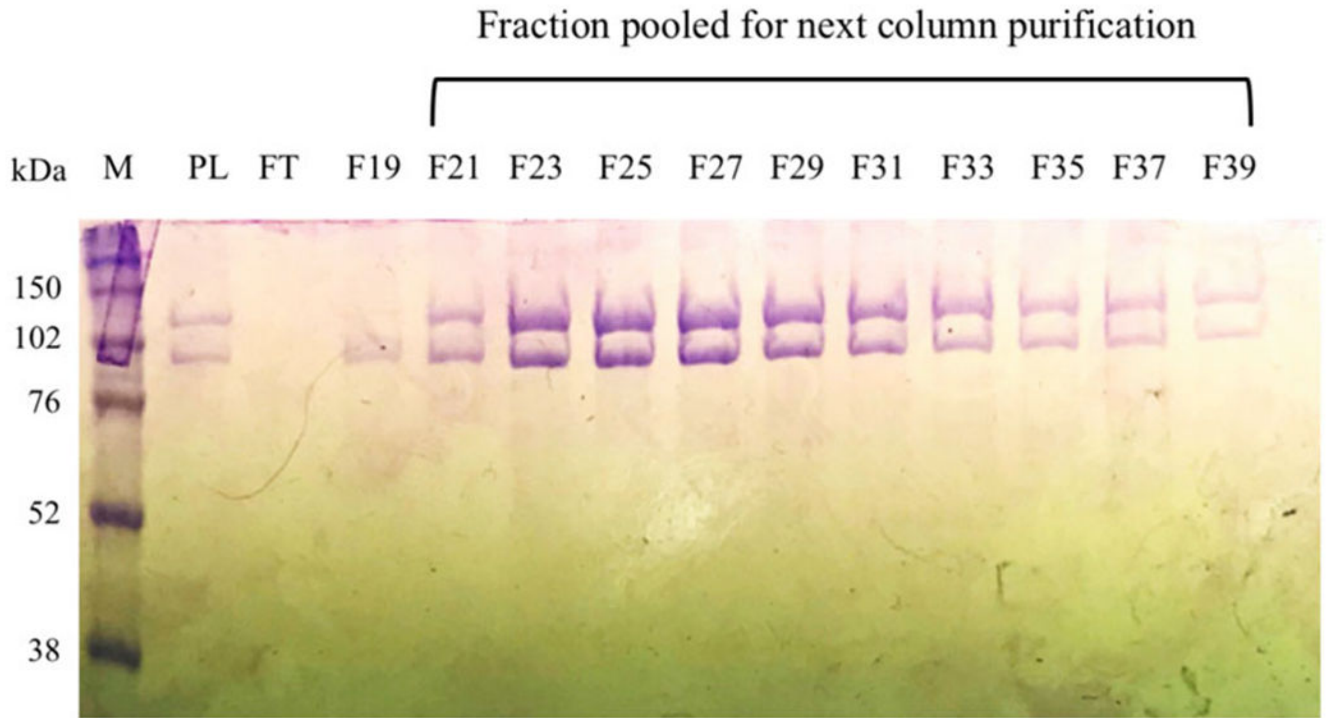

Fig. 4.

Purification of LigIIIα/XRCC1 complex using a dsDNA cellulose column. PL and FT represent pre-load and flow through, respectively. Every other fraction from the peak was analyzed by SDS-PAGE to evaluate protein co-elution as an indicator of complex formation

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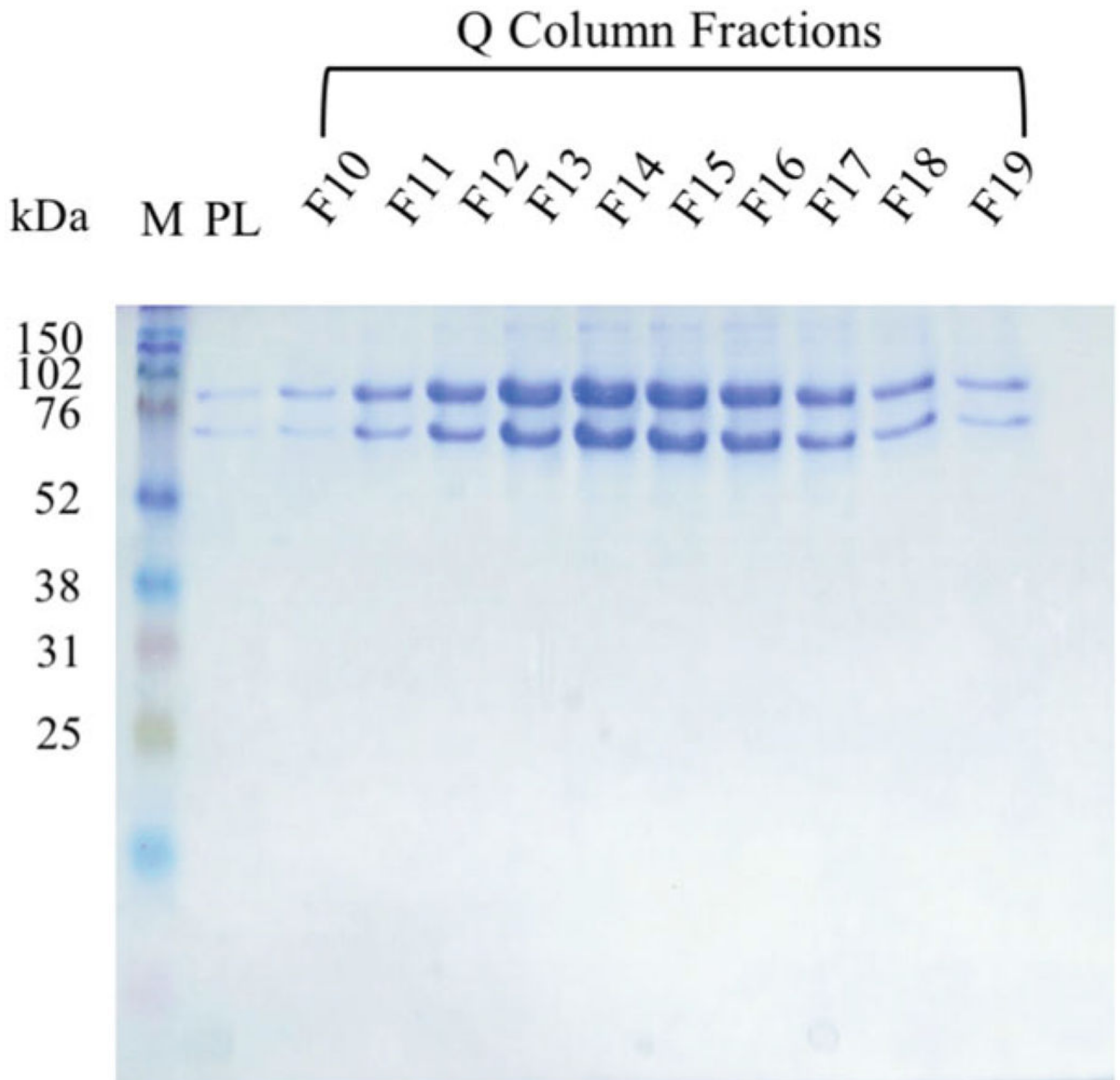

Fig. 5.

Purification of LigIIIα/TDP1 complex using a HiTrap Q column. PL represents pre-load. Fractions from the peak were analyzed by SDS-PAGE to evaluate protein co-elution as an indicator of complex formation

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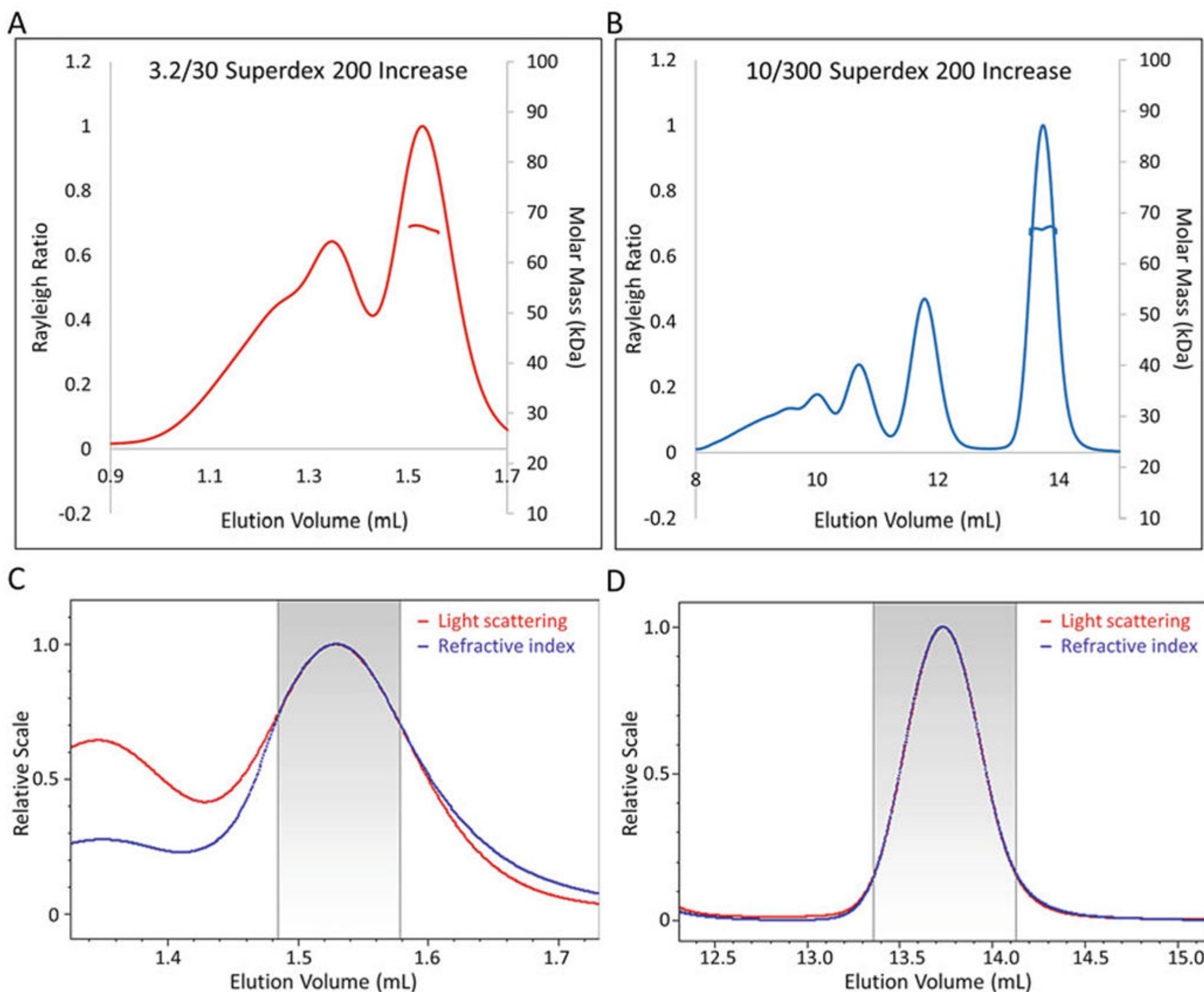

Fig. 6.

BSA injected onto 3.2/30 (**a**, **c**) and 10/300 (**b**, **d**) Superdex 200 Increase gel filtration columns showing poor and ideal peak separation, respectively. Adequate peak separation is necessary for proper band broadening correction. The ASTRA software tries to align light scattering (red) and refractive index (blue) detectors in poor (**c**) and ideal (**d**) peak separation

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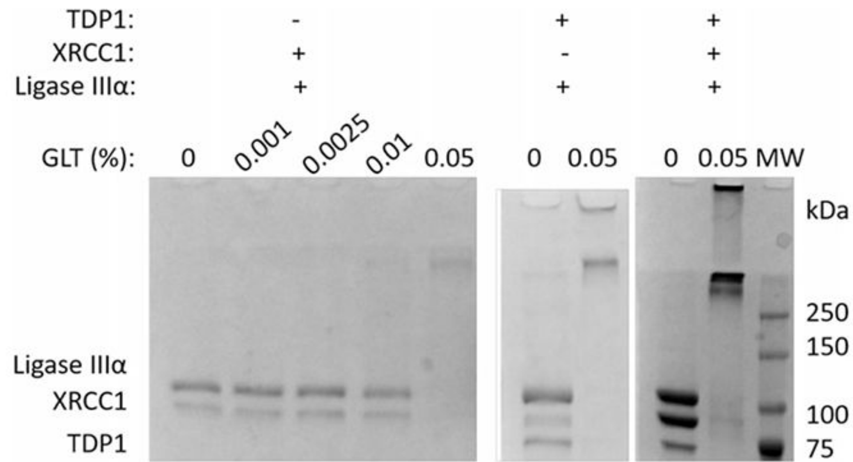

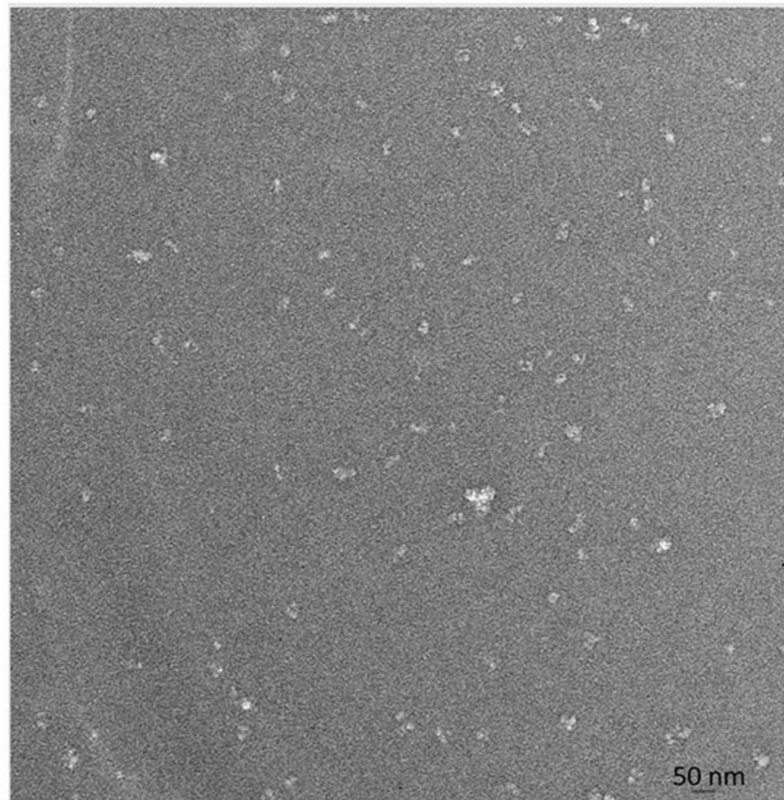

Fig. 7.

(**a**) SDS-PAGE gels of LigIIIα complexed with XRCC1, TDP1, or both. In each gel the samples are shown before and after mild chemical crosslinking with GLT. (**b**) Example negative stain micrograph of LigIIIα crosslinked to TDP1 showing a moderate concentration of particles

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Table 1

Columns used for LigIIIα complex purification

Protein complex	1st column	2nd column	3rd column	4th column
LigIIIα/6His-XRCC1	HisTrap	HiLoad Superdex 200 16/60	dsDNA Cellulose	
LigIIIa/6His-Flag-TDP1	HisTrap	HiLoad Superdex 200 16/60	HiTrap Q	
LigIIIa/6His-XRCC1/Flag-TDP1	HisTrap	HiLoad Superdex 200 16/60	HiTrap Q	HiTrap SP