Published in final edited form as:

Methods Mol Biol. 2012 ; 831: 181–195. doi:10.1007/978-1-61779-480-3_11.

Preparation of the Modular Multi-Domain Protein RPA for Study by NMR Spectroscopy

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Abstract

The integrity and propagation of the genome depend upon the fidelity of DNA processing events, such as replication, damage recognition, and repair. Requisite to the numerous biochemical tasks required for DNA processing is the generation and manipulation of single-stranded DNA (ssDNA). As the primary eukaryotic ssDNA-binding protein, Replication Protein A (RPA) protects ssDNA templates from stray nuclease cleavage and untimely reannealing. More importantly, RPA also serves as a platform for organizing access to ssDNA for readout of the genetic code, recognition of aberrations in DNA, and processing by enzymes. We have proposed that RPA's ability to adapt to such a broad spectrum of multiprotein machinery arises in part from its modular organization and interdomain flexibility. While requisite for function, RPA's modular flexibility has presented many challenges to providing a detailed characterization of the dynamic architecture of the full-length protein. To enable the study of RPA's interdomain dynamics and responses to ssDNA binding by biophysical methods including NMR spectroscopy, we have successfully produced recombinant full-length RPA in milligram quantities at natural abundance and enriched with NMR-active isotopes.

Keywords

Replication Protein A; DNA processing; Protein modularity; Isotopic labeling; Recombinant expression; Protein purification; NMR spectroscopy

1. Introduction

As the primary eukaryotic single-stranded DNA (ssDNA)-binding protein, Replication Protein A (RPA) prevents reannealing of unwound DNA strands, controls access to DNA templates, and serves as a scaffold for the assembly and disassembly of DNA processing machinery (1, 2). A heterotrimer, RPA's three subunits (RPA70, RPA32, and RPA14) contain seven structured domains interconnected by flexible linkers. Three of these domains form the trimeric core of the protein (70C, 32D, 14), from which emanate the flexibly linked N-terminal domains of RPA70 (70N, 70A, 70B), as well as the disordered N-terminal and structured C-terminal domains of RPA32 (32N and 32C, respectively). Binding of ssDNA is facilitated by domains 70A, 70B, 70C, and 32D, which together occupy an occluded site size of 30 nucleotides (1). Interactions with other DNA-processing proteins are primarily mediated by domains 70N and 32C, and the principal DNA-binding domains 70A and 70B (1, 2).

As a universal participant in DNA processing, RPA must interact with a wide array of structurally unique multiprotein complexes. The flexible, modular organization of the protein is thought to be critical for enabling such structural adaptability (3). Although high-resolution X-ray or NMR structures of all individual RPA domains are available (4–8), the dynamic interdomain organization of full-length RPA and the accompanying structural alterations imposed by DNA processing have not been extensively characterized. The full-length protein's intrinsic flexibility poses several challenges to study by X-ray diffraction; and at 116 kDa, RPA falls outside the size limit of conventional NMR methods (30–40 kDa). Application of advanced NMR approaches, however, namely, deuterium labeling and TROSY- or CRINEPT-based techniques, has allowed this size limitation to be extended to proteins in excess of 100 kDa (9–12). This, combined with the discrete distribution of molecular mass among RPA domains (50 kDa for the trimer core and 10–14 kDa for the remaining domains), makes feasible characterization of the full-length protein by NMR (13).

Here, we describe the production of full-length RPA by recombinant expression in *Escherichia coli* and subsequent purification of the protein by a series of FPLC steps. The protocols provided include those required for preparation of ^2H -, ^{15}N -enriched RPA for study by NMR spectroscopy.

2. Materials

2.1. Cell Transformation

1. RPA pET15b plasmid (see Note 1).
2. BL21(DE3) pLysS competent cells: 100- μL aliquots stored at -80°C .
3. LB medium plates: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, 15 g/L agar dissolved in Milli-Q water (filtered to a resistance of 18.3 $\text{M}\Omega\text{-cm}$) and autoclaved at 121°C for 15 min. Add antibiotic stocks (ampicillin and chloramphenicol) at 1:1,000 dilution when the medium has cooled to $50\text{--}60^\circ\text{C}$ (14) (see Note 2).
4. 1,000 $\times$ Ampicillin stock: 100 mg/mL in Milli-Q water, sterilize by filtration at 0.2 μm (see Note 3).
5. 1,000 $\times$ Chloramphenicol stock: 34 mg/mL in ethanol, sterilize by filtration at 0.2 μm (see Note 3).
6. SOC recovery medium: 20 g/L tryptone, 0.5 g/L NaCl, 5 g/L yeast extract, 2.5 mM KCl, 5 mM MgCl_2 , 5 mM MgSO_4 , 20 mM glucose dissolved in Milli-Q water and auto-claved at 121°C for 15 min (14).

2.2. Cell Expression Testing

1. RPA pET15b BL21(DE3) pLysS LB plate (Subheading 2.1).
2. Sterile 10-mL test culture tubes.
3. LB medium: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract dissolved in Milli-Q water and autoclaved at 121°C for 15 min.

¹The tricistronic RPA pET15b vector was a gift from the lab of Alexey Bochkarev. 6 $\times$ -His tags with thrombin cleavage sites precede the RPA70 and RPA14 subunits. The order of subunit open reading frames (ORFs) is as follows: RPA70, RPA14, RPA32.

²Using freshly prepared ampicillin stock in the LB medium is important for ensuring robust RPA transformation. Even though the choice of the BL21(DE3) pLysS cell line is designed to circumvent leaky expression, even small amounts of noninduced RPA can potentially result in resistant cells with less than robust expression.

³Antibiotic stocks may be aliquoted and stored at -20°C for future use. For long-term storage of ampicillin stocks, storage at -80°C is recommended.

4. 1,000× Antibiotic stocks (Subheading 2.1).
5. 1 M IPTG: Sterilize by filtration at 0.2 μm and store at -20°C .
6. 2× SDS loading buffer: 100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS (electrophoresis grade), 0.2% (w/v) bromophenol blue, 20% (w/v) glycerol, 200 mM β -mercaptoethanol (βME , added fresh) (14).
7. 8 M urea.
8. Precast 4–12% Bis-Tris SDS-PAGE gel (Invitrogen).
9. 1× MES SDS running buffer (Invitrogen).
10. 1× Prestained molecular weight standards.
11. SimplyBlue SafeStain.

2.3. Preparation of Culture Media

2.3.1. LB Medium

1. LB medium (Subheading 2.2).
2. 1,000× Antibiotic stocks (Subheading 2.1).
3. 500-mL Erlenmeyer flask with baffles.
4. Six 2.8-L Fernbach flasks with baffles.

2.3.2. Minimal Medium

1. Milli-Q water (900 mL/L medium).
2. 10× M9 salts: 5 g/L NaCl, 30 g/L KH_2PO_4 , 60 g/L Na_2HPO_4 dissolved in Milli-Q water, adjusted to pH 7.4 with 10 M NaOH, and autoclaved at 121°C for 15 min.
3. 1 M MgSO_4 : Sterilize filter at 0.2 μm and store at room temperature.
4. 1 M CaCl_2 : Sterilize filter at 0.2 μm and store at room temperature.
5. 20% (w/v) Glucose: Sterilize filter at 0.2 μm and store at room temperature.
6. 1 M Thiamine hydrochloride: Sterilize filter at 0.2 μm and store at room temperature.
7. 1,000× Antibiotic stocks (Subheading 2.1).
8. $^{15}\text{NH}_4\text{Cl}$.
9. 500-mL Erlenmeyer flask with baffles.
10. Six 2.8-L Fernbach flasks with baffles.

2.3.3. Deuterated Minimal Medium

1. 99% D_2O .
2. Dry components: 0.5 g/L NaCl, 3 g/L KH_2PO_4 , 6 g/L Na_2HPO_4 , 0.24 g/L- MgSO_4 , 11.1 mg/L- CaCl_2 , 2 g/L-glucose, 0.337 g/L thiamine hydrochloride, 0.5 g/L $^{15}\text{NH}_4\text{Cl}$, and 0.1 g/L ampicillin.
3. Six sterile vacuum filtration systems with 1-L storage containers.
4. Six sterile 2.8-L Fernbach flasks with baffles.

2.4. Starter Cultures

2.4.1. LB Medium

1. RPA pET15B BL21(DE3) pLysS LB plate (Subheading 2.1).
2. 250 mL LB starter culture (Subheading 2.3.1).

2.4.2. Minimal Medium and Deuterated Minimal Medium

1. RPA pET15B BL21(DE3) pLysS LB plate (Subheading 2.1).
2. Sterile 10-mL test culture tubes.
3. LB medium (Subheading 2.2).
4. 1,000× Antibiotic stocks (Subheading 2.1).
5. 250 mL Minimal medium starter culture (Subheading 2.3.2).

2.5. Large-Scale Cell Culture and Overexpression

1. Starter culture (Subheading 2.4).
2. 6 L Sterile media in Fernbach flasks (Subheading 2.3).
3. 1 M IPTG (Subheading 2.2).
4. Bleach or 1% Terg-a-Zyme solution for decontamination of spent media.

2.6. RPA Purification

2.6.1. Cell Lysis

1. *Lysis buffer*: Dissolve two complete EDTA-free protease inhibitor cocktail tablets (Roche) in 80 mL of Ni-NTA buffer A (Subheading 2.6.2) in a 150-mL glass beaker on ice immediately prior to use.
2. 100-mL Glass homogenizer.
3. Sonic dismembrator.
4. 25-mm diameter, 0.45- μ m syringe filter.

2.6.2. Ni-NTA Chromatography

1. Refrigerated Äkta FPLC purification system and accessories.
2. *Ni-NTA buffer A*: 20 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM β ME, 10 μ M ZnCl₂, 10 mM imidazole; adjusted to target pH with concentrated HCl, filtered at 0.45 μ m under vacuum, and stored at 4°C (see Notes 4–7).
3. *Ni-NTA buffer B*: 20 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM β ME, 10 μ M ZnCl₂, 300 mM imidazole; adjusted to target pH with concentrated HCl, filtered at 0.45 μ m under vacuum, and stored at 4°C (see Notes 4–7).
4. 25 mL Ni-NTA pre-packed FPLC column (Sigma–Aldrich).

2.6.3. Desalting Exchange and Heparin Chromatography

1. Centrifugal concentrators (15 mL, 30 kDa MWCO).

⁴The 70C domain of RPA contains a zinc-binding motif, for which ZnCl₂ is included in the purification buffers.

⁷Preparation of 1 L of each buffer should provide more than enough for the entire purification.

2. Refrigerated Äkta FPLC purification system and accessories.
3. *Heparin buffer A*: 20 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM β ME, 10 μ M ZnCl₂, 10% glycerol; adjusted to target pH with concentrated HCl, filtered at 0.45 μ m under vacuum, and stored at 4°C (see Notes 6 and 7).
4. *Heparin buffer B*: 20 mM HEPES, pH 7.5, 1 M NaCl, 5 mM β ME, 10 μ M ZnCl₂, 10% glycerol; adjusted to target pH with concentrated HCl, filtered at 0.45 μ m under vacuum, and stored at 4°C (see Notes 6 and 7).
5. HiPrep 26/10 Desalting column (GE Healthcare).
6. HiTrap 5-mL Heparin HP column (GE Healthcare).

2.6.4. Superdex 200 Gel Filtration Chromatography

1. Centrifugal concentrators (15 mL, 30 kDa MWCO).
2. 0.22- μ m centrifugal spin filters.
3. Refrigerated Äkta FPLC purification system and accessories.
4. *Gel filtration buffer*: 20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM β ME, 10 μ M ZnCl₂, 200 mM arginine; adjusted to target pH with concentrated HCl, filtered at 0.45 μ m under vacuum, and stored at 4°C (see Notes 6 and 7).
5. Superdex 200 HR 10/30 column (GE Healthcare).

2.7. Preparation of Samples for NMR

1. Centrifugal concentrators (15 mL, 30 kDa MWCO).
2. 5- and 4-mm NMR tubes.
3. 99% D₂O.

3. Methods

This section describes a protocol for the production of full-length RPA in *E. coli* and its subsequent purification, including preparation of ²H-, ¹⁵N-enriched protein for study by NMR. As a rule, robust expression of full-length RPA in *E. coli* is challenging as RPA is a relatively large protein (>100 kDa) and its ssDNA binding properties are toxic to bacterial cells. Average yields of RPA over-expressed from the pET15b vector range from 17 mg for 6 L of rich LB culture to 4–8 mg of ¹⁵N-enriched RPA for 6 L of minimal medium culture (1–2 NMR samples at approximately 130 μ M concentration and 260- μ L volume). Working with RPA cultures grown in deuterated minimal medium requires patience and careful monitoring, as a deuterated environment is particularly stressful to the bacterial metabolism. Consequently, deuterated cultures take much longer to reach their target induction densities and usually result in a diminished yield of the recombinant protein. Due to the high cost of D₂O and time investment required for growth and expression, we found it beneficial to pilot a small-scale test culture (100 mL) to develop an expected timeline for the growth and to ensure that all reagents were functioning as expected. Subsequent purification of this culture allowed us to determine that the overall yield of RPA had not suffered significantly from production in a deuterated environment. In the expression protocol below, we describe the full 6 L production run; however, when embarking upon deuterium labeling for the first time, we highly recommend starting with the smaller pilot culture.

⁶ β ME is added fresh to each buffer immediately prior to use. Buffers can be prepared without β ME and stored at 4°C if their use is anticipated to last beyond 1–2 days.

As RPA is a trimeric, DNA-binding protein, the purification protocol below is designed to ensure samples free of contaminating ssDNA, as well as uniform stoichiometry among all three RPA subunits. Expression of RPA from the pET15b vector results in an excess of the RPA70 subunit, which can be successfully separated from the intact heterotrimer with heparin and gel filtration chromatography. The heparin purification step also selects for RPA free from ssDNA contamination.

3.1. Cell Transformation

Ensuring robust antibiotic selection of the RPA vector on solid medium is vital to enabling the success of subsequent liquid cultures (see Note 2).

1. Thaw 100 μ L of BL21(DE3) pLysS competent cells on ice, gently combine with 100 ng of RPA pET15b vector, and incubate for 30 min on ice.
2. Heat shock the cells at 42°C for 45 s and incubate on ice for 2 min. Add 900 μ L of sterile SOC recovery medium.
3. Incubate the cells for 1 h at 37°C and 200–230 rpm, then centrifuge the cells at $16,100 \times g$ for 1 min at room temperature (see Note 8). Remove 900 μ L of the clarified SOC medium and gently resuspend the cells in the remaining medium prior to plating on an LB medium plate.
4. Incubate the plates overnight at 37°C.

3.2. Cell Expression Testing

Expression testing allows confirmation of the expression capability of the transformed bacterial colonies prior to scaling up protein production. The testing also allows for selection of colonies with the most robust expression.

1. Prepare five LB test cultures as follows: Transfer 5 mL of LB medium to a 10-mL sterile culture tube and add 1:1,000 dilutions of ampicillin and chlormaphenicol antibiotic stocks. Inoculate each culture with a colony selected from the center of a freshly transformed RPA pET15b LB plate (see Note 9) and incubate the cultures at 37°C, 200–230 rpm, until they reach an A_{600} of 0.5–0.6 (approximately 3–4 h).
2. Transfer 250 μ L of each LB test culture into an Eppendorf tube as a preinduction sample. Centrifuge the sample at $16,100 \times g$ for 1 min, decant the supernatant, add 7 μ L each of 2 $\times$ SDS-PAGE loading buffer and 8 M urea, and vortex to mix.
3. Add IPTG to a final concentration of 1 mM to the remainder of the test culture to induce expression and continue to incubate with shaking at room temperature for 3 h. Collect a final 250 μ L postinduction sample and process as in step 2.
4. Boil pre- and postinduction SDS-PAGE samples for 5–10 min to denature the lysates and load 2–4 μ L from each sample into a precast 4–12% Bis–Tris SDS-PAGE gel preloaded into an electrophoresis cell filled with 1 $\times$ MES SDS running buffer. Reserve one lane for 5 μ L of 1 $\times$ prestained molecular weight standards. Run the gel at 200 V.
5. Remove the gel from the electrophoresis cell and place in a loosely capped container filled with Milli-Q water (see Note 10). Fix the gel by heating on a high

⁸As the transformation efficiency of the RPA pET15b vector is low and the double-antibiotic selection with the BL21(DE3) pLysS strain is quite stringent, plating the entire transformation culture is recommended.

⁹When transferring a selected colony to the test expression culture, be sure to leave a portion behind for future inoculation of the large-scale cultures. If the colony is too small to divide in this manner, the plate may be left at room temperature for half a day to allow the colony to regrow.

setting in a microwave oven for 1 min, followed by 1 min of cooling. Exchange the water and repeat. Remove the final rinse and stain with SimplyBlue SafeStain for 20 min. Remove the stain and refill the container with deionized water to destain the gel.

6. The relative proportion of RPA32 and RPA14 subunits is too low to observe on the gel. RPA70 should be just distinguishable at the appropriate molecular weight in lanes containing postinduction samples. Select colonies exhibiting the most abundant RPA production for subsequent large-scale expression.

3.3. Preparation of Culture Media

This medium serves for the production of unlabeled RPA. Preparation should include a 250-mL starter culture to accommodate a 6-L large-scale culture.

3.3.1. LB Medium

1. Dissolve LB components (Subheading 2.2) in Milli-Q water in a 500-mL baffled Erlenmeyer flask (starter culture) or 2.8-L baffled Fernbach flasks (large-scale culture), autoclave at 121°C for 15 min, and cool to 50–60°C.
2. Add ampicillin and chloramphenicol at 1:1,000 dilution immediately prior to inoculation.

3.3.2. Minimal Medium—This medium serves for the production of ¹⁵N-enriched RPA. Preparation should include a 250 mL starter culture to accommodate a 6 L large-scale culture. A 250 mL minimal medium culture also serves as an adaptation culture for production of deuterated protein.

1. Dilute 10× M9 salts in Milli-Q water to 1× in a 500-mL baffled Erlenmeyer flask (starter culture) or 2.8-L baffled Fernbach flasks (large-scale culture), autoclave at 121°C for 15 min, and cool to 50–60°C.
2. Add the following components immediately prior to inoculation: 0.5 g/L of ¹⁵NH₄Cl (see Note 11), 2 mL/L of 1 M MgSO₄, 100 μL/L of 1 M CaCl₂, 10 mL/L of 20% glucose, 1 mL/L of 1 M thiamine hydrochloride, and antibiotic stocks at 1:1,000 dilution.

3.3.3. Deuterated Minimal Medium—This medium serves for six 1 L large-scale production cultures and is prepared immediately prior to inoculation after the success of the 250 mL minimal medium adaptation culture has been ascertained.

1. Dry autoclave six 2.8-L Fernbach flasks with baffles and allow to dry thoroughly overnight.
2. Dissolve all dry components in 6 L of 99% D₂O (see Notes 12 and 13) and immediately sterilize the medium in 1-L batches by using sterile vacuum filtration systems (i.e., 1 L/unit). This apparatus filters the medium directly into a sterile 1-L

¹⁰An empty gel tip box will suffice. The level of water should be enough to immerse the gel.

¹¹The ¹⁵NH₄Cl is measured out and added directly to the sterile medium as a powder. This ensures that the labeled material is not wasted should a step prior to the inoculation fails.

¹²As mentioned at the beginning of Subheading 3, the volume of deuterated minimal medium may be adjusted for small-scale testing.

¹³Mixing of the deuterated minimal medium dry components should take place in a clean, *dry* container and be carried out as efficiently as possible to prevent exchange with ambient water vapor. If a container large enough to accommodate 6-L volume is not available, substitution of two 3-L containers with subsequent exchange and mixing between the two batches can be used to ensure the homogeneity of the medium across all cultures.

bottle. Chloramphenicol is *not* included in the medium at this stage to ease the metabolic burden on the cells.

3. Carefully transfer each 1 L of sterile deuterated minimal medium to a dry, sterile Fernbach flask (see Note 14).

3.4. Starter Cultures

3.4.1. LB Medium

1. Inoculate a 250 mL LB starter culture (Subheading 3.3.1) directly with an RPA pET15b colony selected from the test expression.
2. Grow the culture overnight at 37°C, 200–230 rpm. The culture should be cloudy in the morning.

3.4.2. Minimal Medium and Deuterated Minimal Medium

1. Inoculate a 4 mL LB starter culture (4 mL LB + 1:1,000 ampicillin/chloramphenicol in a 10-mL culture tube) with an RPA pET15b colony selected from the test expression. Grow for 3–4 h at 37°C, 200–230 rpm, or until cloudy.
2. Inoculate the 250 mL minimal medium starter culture (Subheading 3.3.2) with the 4 mL LB starter culture and shake overnight at 37°C. The culture should be cloudy in the morning.

3.5. Large-Scale Cell Culture and Overexpression

1. Prepare six 1 L cultures of rich LB medium, minimal medium, or deuterated minimal medium as described above (Subheading 3.3) and inoculate each with 30 mL (40 mL for deuterated minimal medium) of the corresponding overnight starter culture.
2. Grow the cultures at 37°C, 200–230 rpm, until an A_{600} of 0.6–0.7 is reached (see Note 15).
3. Allow the cultures to equilibrate for half an hour with agitation at 18°C (or room temperature for deuterated minimal medium) prior to induction. Collect a preinduction SDS-PAGE sample as described above (Subheading 3.2) and induce the cells with 1 mM IPTG. Allow cells to express overnight (approximately 16–18 h).
4. The A_{600} at the end of the expression period should be 1.8–2.0 for LB medium cultures and 0.9–1.0 for standard and deuterated minimal media cultures. Collect postinduction SDS-PAGE samples from the cultures as described above (Subheading 3.2). Harvest the cultures by centrifuging at $10,000 \times g$ for 20 min at 4°C.
5. Decant the supernatant and reserve the spent deuterated media for recycling (15). Spent LB or minimal media may be decontaminated by the addition of bleach or a 1% Terg-a-zyme solution for 30 min, and then discarded. If purification does not follow immediately, transfer the pellets to sterile 50-mL conical tubes and freeze at

¹⁴Performing this sterile transfer in the presence of a Bunsen burner flame is advised.

¹⁵In our experience, growth in rich LB medium requires 3–4 h to reach the target A_{600} while growth in minimal medium requires 8–10 h. For deuterated minimal medium, this time-line extends to 1.5–2 days. To ensure that the induction occurred during daylight hours, cultures were switched to agitation at 20°C overnight after a full day of growth at 37°C and then returned to 37°C the next morning. The target A_{600} was reached during the afternoon of the second day.

–80°C. Run pre- and postinduction SDS-PAGE samples as in Subheading 3.2 to confirm the presence of RPA expression.

3.6. RPA Purification

Purification of RPA involves three primary steps: Ni-NTA affinity, heparin, and size-exclusion chromatography. For best results, the protocol should be completed over the course of 2 days, where Ni-NTA and heparin chromatography steps are accomplished the first day and the final gel filtration step is carried out on the second day. If necessary, the Ni-NTA and heparin steps may be divided into two separate days and protein fractions from each purification kept at 4°C overnight. Ideally, though, the time from cell lysis to the final gel filtration exchange should be kept to a minimum.

3.6.1. Cell Lysis

1. If cells have been frozen at –80°C, thaw the pellets by submerging the 50-mL conical tubes in cool water. Meanwhile, prepare and chill the lysis buffer and pre-chill the 100-mL glass homogenizer on ice (see Note 16).
2. Transfer all 6 L of RPA cell pellets into the 100-mL homogenizer, rinse the 50-mL conical tubes with ice-cold lysis buffer, and add the rinse and any remaining lysis buffer to the homogenizer.
3. Homogenize the lysate until smooth (10–15 strokes).
4. Return the lysate to the 150-mL glass beaker and pack this into a 2-L plastic beaker filled with an ice-water bath (see Note 17). Ensure that there is sufficient ice to securely brace the beaker and prevent floating.
5. Sonicate the lysate with a macrotip set at 60% power for 5.0 min of total process time (pulsing 5.0 s on and 5.0 s off). Pause the sonicator half-way through this cycle to replenish the ice-water bath and to check the temperature of the lysate (see Note 16). The lysate should become translucent and less viscous as the sonicator disrupts the cellular material. If the lysate viscosity remains unchanged after the cycle is complete, repeat the cycle once more, monitoring the ice-water bath and lysate temperature.
6. Clarify the lysate by centrifuging at $48,000 \times g$ for 20 min at 4°C. Ensure that both centrifuge and rotor are pre-chilled to at least 4°C.
7. Decant and filter the clarified supernatant through a 0.45- μ m membrane. Store on ice for immediate loading onto the FPLC Ni-NTA column.

3.6.2. Ni-NTA Chromatography—Steps 2–4 are implemented as a pre-programmed Äkta FPLC method.

1. Equilibrate the prepacked 25 mL Ni-NTA column with three column volumes (3 CVs) each of filtered Milli-Q water and Ni-NTA buffer A (see Note 18).
2. Load the filtered lysate onto the equilibrated Ni-NTA column at 1.0–1.5 mL/min.

¹⁶RPA is susceptible to proteolytic cleavage, particularly at the unstructured 60–70 amino-acid linker that connects the 70N and 70A domains. Throughout the purification, it is essential that all buffers are kept ice cold and that heating from other steps of the lysis (sonication, centrifugation) is kept to a minimum.

¹⁷The ice-water bath serves as a heat sink during sonication of the lysate.

¹⁸Initializing the FPLC system (pump washing, cleaning super-loops, setting up fraction collectors), as well as equilibration of the Ni-NTA column, should occur prior to or concurrently with cell lysis to ensure that the clarified, filtered lysate can be loaded directly into the system as soon as it is available.

3. Wash unbound lysate from the column with 4 CVs of Ni-NTA buffer A at 2.5 mL/min.
4. Elute RPA with a 4 CV gradient (0–100% Ni-NTA buffer B, 10–300 mM imidazole), collecting 6-mL fractions at 2.5 mL/min.
5. Assess the presence of RPA from the A_{280} chromatogram trace and SDS-PAGE of relevant fractions (sampling 5 μ L of each fraction). Pool fractions containing all three RPA subunits for further processing (typically, a 60-mL pool).

3.6.3. Desalting Exchange and Heparin Chromatography—The charged DNA-binding clefts of RPA render it sensitive to the absence of ambient salt. Effective binding of RPA to the heparin matrix, however, requires a low salt content in the loading buffer. Direct dialysis into the loading buffer (heparin buffer A) usually provokes extensive precipitation of RPA. Buffer exchange by desalting, however, allows for the rapid and successful transfer of RPA into the loading buffer with minimal aggregation. Once the series of FPLC desalting runs are complete, it is imperative to load the exchanged protein directly onto the heparin column to restore a stabilizing ionic environment. As before, loading, washing, and elution are implemented automatically using pre-programmed Äkta FPLC methods.

1. Pre-rinse two centrifugal concentrators (15 mL, 30 kDa MWCO) with Milli-Q water by centrifuging at $3,700 \times g$ for 10 min at 4°C. Concentrate the Ni-NTA RPA pool to ~30 mL and store on ice for desalting into heparin buffer A (see Notes 19 and 20).
2. Equilibrate the HiPrep 26/10 Desalting column with 2 CVs of filtered Milli-Q water and 1.5 CVs of heparin buffer A. Equilibrate the HiTrap 5 mL Heparin HP column with 5 CVs of filtered Milli-Q water and 3 CVs of heparin buffer A (see Note 21).
3. Filter and load 10 mL of the Ni-NTA RPA concentrate onto the desalting column at 2.0 mL/min, collecting 4-mL fractions. The protein should elute within the first four fractions (16 mL) of the run. Re-equilibrate the column and repeat the run twice for the remaining 20 mL of RPA Ni-NTA concentrate. Store fractions on ice until all runs are complete.
4. Combine all three desalting pools (48 mL), filter at 0.45 μ m, and load directly onto the equilibrated heparin column at 1.0 mL/min.
5. Wash out unbound sample with 3 CVs of heparin buffer A at 2.5 mL/min.
6. Elute RPA with a 20 CV gradient (0–100% heparin buffer B, 50 mM to 1 M NaCl), collecting 4-mL fractions at 2.5 mL/min.
7. Assess the presence of RPA from the A_{280} chromatogram trace and SDS-PAGE of relevant fractions (sampling 5 μ L of each fraction). The elution should include two major peaks: the first corresponding to RPA70 exclusively and the second to trimeric RPA. Fractions containing all three RPA subunits are pooled for further final purification (typically, a 40-mL pool).

¹⁹Centrifugal concentrators are usually spun in 10–15-min increments and carefully mixed with each addition of the Ni-NTA pool to prevent buildup and aggregation of RPA at the base of the concentrator.

²⁰The desalting resolution of the HiPrep 26/10 Desalting column (GE Healthcare) is 10 mL; that is, the column can effectively exchange 10 mL of injected sample into the target buffer without contamination from the original buffer. To avoid desalting too concentrated a volume of RPA and triggering aggregation, the Ni-NTA pool is processed in three sequential 10-mL batches.

²¹As with the Ni-NTA step, initializing the FPLC system and equilibrating the desalting and heparin columns should occur prior to or concurrently with concentrating the RPA Ni-NTA pool to allow for immediate loading once the target volume is reached.

3.6.4. Superdex 200 Gel Filtration Chromatography—Gel filtration provides a final polishing step for the purification and ensures the removal of any trace RPA70 or low-molecular-weight contaminants. As before, loading and elution are implemented automatically using a pre-programmed Äkta FPLC method.

1. Equilibrate the Superdex 200 HR 10/30 column with 1.5 CVs of filtered Milli-Q water and 1.5 CVs of gel filtration buffer.
2. Pre-rinse two centrifugal concentrators (15 mL, 30 kDa MWCO) with Milli-Q by centrifuging at $3,700 \times g$ for 10 min at 4°C. Concentrate the heparin RPA pool to ~300–500 μ L (see Note 19).
3. Filter the concentrate by using a 0.22- μ m centrifugal spin filter in a refrigerated (4°C) centrifuge and load onto the Superdex 200 HR 10/30 column at 0.3 mL/min, collecting 0.5-mL fractions for 1.5 CVs.
4. As before, assess the presence of RPA from the A_{280} trace and SDS-PAGE of relevant fractions (sampling 5 μ L of each fraction).
5. Before the final RPA fractions are pooled, acquire final A_{280} and A_{260} measurements by UV-Vis spectrophotometry to ensure that the selected fractions are DNA-free, as determined by A_{260}/A_{280} ratios of 0.64 or less.

3.7. Preparation of Samples for NMR

1. Pre-rinse two centrifugal concentrators (15 mL, 30 kDa MWCO) with Milli-Q water by centrifuging at $3,700 \times g$ for 10 min at 4°C. Concentrate the S200 RPA pool to ~500–600 μ L (see Note 19), monitoring the protein concentration by UV-Vis spectrophotometry (the gel filtration buffer may serve as a blank).
2. The target NMR concentration for RPA is ~100–130 μ M (10–15 mg/mL) with a minimum sample volume of 260 μ L (using a 4-mm-diameter NMR tube). Once the target concentration is reached, centrifuge 260–300 μ L of the concentrate at $16,100 \times g$ for 5 min at 4°C to remove any stray precipitation.
3. Load the protein into a standard 4-mm diameter NMR tube, which may be fitted with an adaptor to fit a 5-mm spinner or slipped into a 5-mm diameter tube containing 120 μ L D₂O without the need for the adaptor.

Acknowledgments

The authors would like to thank Dr. Dalyir Pretto and Susan Meyn. This work was supported by the National Institutes of Health operating grant R01 GM65484 and graduate training grant T32 GM08320.

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